Human immunodeficiency virus type 1 Tat increases cooperation between AP-1 and NFAT transcription factors in T cells

Alicia M. Hidalgo-Estévez, Esther González, Carmen Punzón and Manuel Fresno

Correspondence
Manuel Fresno
mfresno@cbm.uam.es

Centro de Biología Molecular ‘Severo Ochoa’ (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

INTRODUCTION

The pathogenic mechanisms underlying human immunodeficiency virus type 1 (HIV-1) infection are extremely complex, and virological as well as immunological factors may contribute to pathogenesis (Rowland-Jones, 2003; Stevenson, 2003). Among the viral factors involved, the protein Tat has been studied widely. Tat is a small (72–101 aa) regulatory viral protein required for efficient transcription and virus replication (Jeang et al., 1994; Vacca et al., 1994; Vacca et al., 1995). Tat protein Tat has been shown to interact with several members of the transcriptional machinery during the process of initiation and elongation of viral transcription (Cullen, 1998). Furthermore, Tat not only affects viral transactivation but also mediates alterations of multiple cellular processes. Among these, Tat has been involved in the aberrant expression of several cytokine and chemokine genes such as tumour necrosis factor α (Buonaguro et al., 1992), transforming growth factor β (Zauli et al., 1992), interleukin 6 (IL-6) (Ambrosino et al., 1997), IL-2 (Vacca et al., 1994; Westendorp et al., 1994), gamma interferon (Zagury et al., 1998), IL-8 (Ott et al., 1998), monocyte chemoattractant protein 1 (MCP-1) (Abraham et al., 2005) and MCP-2 (Izmailova et al., 2003). Many of these effects are most likely mediated by alterations of gene expression by Tat. In this regard, a direct interaction of the viral protein with several transcription factors including Oct, Sp1 (Jeang et al., 1993), Egr (Yang et al., 2002), E2F (Ambrosino et al., 2002) and nuclear factor of activated T cells (NFAT) (Macián & Rao, 1999) has been demonstrated. In addition, indirect mechanisms have been proposed to explain alterations in the activity of NF-κB (Biswas et al., 1995; Demarchi et al., 1999) and activator protein 1 (AP-1) (Li et al., 1997; Kumar et al., 1998) transcription factors.

Cytokine transcription is controlled by, among others, the transcription factors NF-κB, AP-1 and NFAT (Ghosth et al., 1998; Macián et al., 2001). AP-1 dimers are composed of members of the Jun, Fos or ATF families. Their activation is regulated by transcriptional and post-transcriptional mechanisms, including specific interactions with transcriptional co-activators (Karin et al., 1997). In contrast to AP-1, NFAT proteins are functional as monomers and their activation is tightly regulated by intracellular calcium concentrations. Thus, the phosphatase calcineurin, which dephosphorylates NFAT inducing its translocation to the nucleus and increasing its DNA binding and transactivating activities, is activated by calcium increases (Im & Rao, 2004).

The interplay between families of transcription factors represents an additional mechanism of gene regulation. Interactions between NFAT and AP-1 proteins take place on composite elements present in, among others, the IL-2, IL-4, IL-5, CD40L and granulocyte–macrophage colony-stimulating factor (GM-CSF) and cyclo-oxygenase 2 (COX-2) promoters (Kel et al., 1999; Íñiguez et al., 2000; Macián et al., 2001; Johnson et al., 2004). The NFAT distal site of the human IL-2 promoter allowed the identification of the
NFAT/AP-1 binding complex (Shaw et al., 1988). These cooperative interactions have an important functional significance, as they allow the integration of signals by the binding of different factor (Rel-ZIP) transcription factors to specific DNA-response elements. They increase the functional diversity of the transcription factors, as these supracomplexes show DNA-binding and transactivating capacities distinct from either transcription factor partner alone (Nolan, 1994).

As the Tat viral protein has been shown to affect cytokine transcription and NFAT, we considered it to be of interest to study how this viral protein can affect its functional cooperation with AP-1. Here, we have shown that Tat interacts with NFAT, favouring its association with c-Jun at composite NFAT/AP-1 elements and increasing its transactivating activity. Furthermore, we showed that Tat strongly increases NFAT/AP-1 cooperative DNA binding and transactivation without having any significant effect on the individual binding of these factors to DNA.

**METHODS**

**Cell cultures and reagents.** Jurkat Jhan cells (both wild-type and stably expressing the full-length HIV-1 Tat of 86 aa) have been described previously (González et al., 2001). Cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 25 ng ml⁻¹; Sigma Chemicals) and calcium ionophore A23187 (Io, 1 μM; Sigma Chemicals) as indicated. None of the agents affect the viability of the cells at the concentrations used.

**Plasmid constructs.** The reporter plasmid pLTR-luc, containing the long terminal repeat (LTR) from HIV-1 subtype B, was a generous gift from Dr I. L. Virelizier (Schwartz et al., 1990). The plasmid pB-CONA-luc contained three tandem copies of the κB site of the immunoglobulin γ chain promoter cloned upstream of the calmodulin (CONA) transcription start site (Arenzana-Seisdedos et al., 1993). The pFNAT-luc reporter plasmid was a gift from Dr Crabtree (Durand et al., 1988) and contained three tandem copies of the NFAT/AP-1 distal site of the human IL-2 promoter fused to the minimal human IL-2 promoter. The p–73-col-luc plasmid included the AP-1-responsive regions (–73/+63 bp) of the human collagenase promoter fused to the luciferase gene (Deng & Karin, 1993). The p275-Cox-2-luc plasmid containing the –170 to +104 region of the human Cox-2 promoter fused to the luciferase gene and their AP-1 and NFAT mutated versions have been described previously (Iñiguez et al., 2000). The plasmid pEF-BOS-NFATc2 contained the gene encoding the influenza virus haemagglutinin (HA)-tagged NFATc2 (NFAT1) and was a generous gift from Dr J. M. Redondo (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain), whilst pSH107-NFATc1 (NFAT2) was a gift from Dr G. Crabtree. RSV-c-Jun was provided by Dr A. Muñoz (Instituto de Investigaciones Biomédicas ‘Alberto Sols’, Madrid, Spain) and HA-tagged pCMV-c-Jun-HA by Dr C. Weiss (Weiss et al., 2003). pCMV-Tat was a gift from Dr J. Alcami (Instituto de Salud Carlos III, Madrid, Spain) and contained full-length HIV-1 Tat (86 aa) under the control of the cytomegalovirus (CMV) immediate-early promoter (Schwartz et al., 1990). pCDNA3-Tat plasmids carrying the Y26A and Y47N mutations were a generous gift of Dr B. Berkhour (Verhoef & Berkhour, 1999). The pCDNA3 plasmid (Invitrogen) was used as a control in the transfection of expression plasmids or to adjust the quantities of DNA transfected. The promoter region of IL-4 containing a pure NFAT site (pIL-4-luc) was a gift from Dr R. Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, USA) (Aune & Flavell, 1997). Tat-HA was generated from pSV2-tat72 (obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH) from Dr A. Franken (Frankel & Pabo, 1988). The plasmid pRL-tk-luc (Promega) was used to evaluate transfection efficiency.

Plasmid pGEX2TK-Tat was obtained from E. Muñoz (Universidad de Córdoba, Spain). Purification of the GST–Tat fusion protein was performed as described previously (Blazquez et al., 1999). The purity and integrity of the proteins were routinely checked by Western blotting, using the anti-Tat polyclonal antiserum.

**Western blot analysis.** Nuclear extracts of Jurkat cells and total extracts of COS-7 cells transfected with the various plasmids obtained as described previously (González et al., 2001) were separated by SDS-PAGE. For the detection of NFATc2 and c-Jun, 6% polyacrylamide gels were electrophoretically transferred to nitrocellulose filters (Bio-Rad); for the detection of Tat, 17% polyacrylamide gels were transferred to ProBlott PTMP membranes (Applied Biosystems) and processed as described previously (González et al., 2001). The anti-NFATc2 672 serum (a generous gift from Dr J. M. Redondo) was used at a 1:3000 dilution and the anti-c-Jun (Santa Cruz Biotechnology), anti-HA antibodies, anti-Tat serum (obtained through Dr B. Cullen, AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; Hauber et al., 1987) and the monoclonal antibody against HIV-1 Tat (aa 1–9) (Advanced Biotechnologies) were diluted 1:1000.

**Electrophoretic mobility-shift assays (EMSA).** Nuclear extracts were obtained from Jurkat cells or from COS-7 cells transfected with pSH107-NFATc1, RSV-c-Jun and/or Tat-HA and gel retardation assays were performed as described previously (Navarro et al., 1998). The sequences of the oligonucleotides used as probes in EMSAs were: 5′-gatcGGAGGAAAAACTGTTTCATACAGAAGGCGT-3′ (distal NFAT site of the human IL-2 promoter); 5′-gatcGCCCAAAGGAA- AATTTGTTTCATACAG-3′ (distal NFAT site of the murine IL-2 promoter); 5′-gatcATAAAATTTCCTGAAAA-3′ (mouse P sequence of the IL-4 promoter); and 5′-CGCTTGATGATGCAGCCGGAAA-3′ (AP-1 consensus oligonucleotide). The lower-case letters indicate the restriction enzyme site Bsp143I, which was added to the promoter sequence.

The identity of the retarded complexes was determined by supershift assays with specific anti-NFATc1 serum 676 (a gift from Dr J. M. Redondo), anti-c-Fos family sera (González et al., 2001) or anti-c-Jun (Santa Cruz Biotechnology).

**Luciferase assays.** Transcriptional activity in Jurkat cells (2 × 10⁶) was measured in reporter gene assays after transient transfection of cells with 0.5 μg luciferase reporter plasmid together with 10 ng pRL-tk-luc in a final volume of 1 ml OptiMem (Iñiguez et al., 2000). Cells were treated for 6 h and then harvested and lysed, and the luciferase activity was measured in a luminometer following the instructions in the Dual-luciferase Assay System kit (Promega). Data are represented in relative firefly luciferase units normalized by the relative Renilla luciferase units obtained in the control samples of each transfection (RLU/ren). Every experiment was carried out in duplicate.

**Immunoprecipitation assays.** For the immunoprecipitation assays, COS-7 cells were transfected with 5 μg pEF-BOS-NFATc2 and/or pCMV-Tat DNA as described previously (González et al., 2001). Cell extracts were separated by SDS-PAGE for Western blot detection of overexpressed proteins and the same extracts were used for the immunoprecipitation assays. Cellular extracts were supplemented with BSA (1 mg ml⁻¹, essentially γ-globulin-free; Sigma Chemicals) and incubated overnight at 4°C with anti-NFATc2 672 serum (1:200 dilution). Protein A-Sepharose (Sigma Chemicals) was
added to the extracts and after 2 h of incubation at 4 °C, immune complexes were pelleted and washed five times with 15 ml lysis buffer each. Immunoprecipitates were analysed by Western blotting.

**GST pull-down assays.** For GST pull-down assays, transfected COS-7 cells were lysed in ice-cold buffer [20 mM HEPES/KOH (pH 7.9), 120 mM KCl, 1 mM MgCl₂, 17% (v/v) glycerol, 1 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.5 mM PMSF, 2 μg pepstatin ml⁻¹, 2 μg leupeptin ml⁻¹, 2 μg aprotinin ml⁻¹, 10 mM NaN₃, 10 mM MnSO₄] for 30 min. The extracts were pre-cleared with GST adsorbed to glutathione–Sepharose (Amersham Biosciences) before affinity chromatography with GST–Tat adsorbed to glutathione–Sepharose for 2 h at 4 °C. Complexes were washed four times in lysis buffer (without NP-40) and analysed by Western blotting.

All of the experiments shown are representative of at least three experiments performed in order to guarantee the reproducibility of the results.

**RESULTS**

**Tat expression in T cells increases transactivation of the NFAT/AP-1 composite element**

Since Tat affects cytokine transcription, we tested its effect on the activation of NFAT, AP-1 and NF-κB transcription factors, which are involved in the regulation of cytokine transcription in T cells. Jurkat cells [either wild-type or stably expressing Tat (Jurkat-Tat⁺)] (González et al., 2001) (Fig. 1b) were transiently transfected with plasmids expressing the luciferase gene under the control of target sequences of these transcription factors. Transfected cells were then stimulated with PMA and Io alone or in combination. As expected, PMA increased NF-κB- and AP-1-dependent promoters (Fig. 1a). Io alone did not induce the NF-κB, AP-1 and NFAT/AP-1 sites, whereas it weakly activated the IL-4 promoter, which is dependent on a pure NFAT site (Aune & Flavell, 1997). Combined treatment with PMA/Io was required to activate the NFAT/AP-1 reporter. Next, we compared transcription induction by these stimuli in Jurkat cells stably expressing Tat with wild-type cells. As expected, in Tat-expressing cells, LTR transcription was strongly increased. Transcription dependent on NF-κB or AP-1 sites was not significantly altered in Jurkat-Tat⁺ cells. In contrast, transcription dependent on the NFAT/AP-1 site was enhanced ~ twofold in Jurkat-Tat⁺ cells after stimulating the cells with a potent stimulus such as PMA/Io (Fig. 1a). These differences were not due to altered expression of NFAT and c-Jun in Tat-expressing cells (Fig. 1b).

To corroborate that this effect was due exclusively to Tat and not to an artefact produced during the generation of stable transfected cells, Jurkat wild-type cells were transiently transfected with a Tat expression plasmid together with various reporter constructs. The expression of low amounts of Tat plasmid induced a very strong upregulation of the HIV-1 LTR promoter and also potentiated the effect of PMA/Io. Tat transfection also resulted in a significant dose-dependent increase in transcription driven by the NFAT/AP-1 composite element over that induced by PMA/Io.

![Fig. 1](http://vir.sgmjournals.org) Transcription factor-mediated transactivation in Jurkat cells constitutively expressing Tat. (a) Transactivation controlled by the transcription factors mentioned on the top of each diagram was evaluated by transiently transfecting Jurkat-Tat⁻ and Jurkat-Tat⁺ cells stably expressing Tat with the luciferase reporter plasmids pxB-CONA-luc, pNFAT-luc, p-73col-luc (for NF-κB, NFAT/AP-1 and AP-1 evaluation, respectively), pLTR-luc or pIL-4-luc together with pRL-tk-luc to normalize transfection efficiency. Cells were cultured in the absence of stimulation (control) or treated with PMA and/or Io for 6 h before lysis and luciferase activity was evaluated. (b) Expression of NFAT, c-Jun and Tat in Jurkat-Tat⁻ and Jurkat-Tat⁺ cells analysed by Western blotting, using anti-NFATc2, anti-c-Jun sera and anti-Tat monoclonal antibody.
However, it did not affect AP-1 transactivation, thus confirming the data obtained in Jurkat cells stably expressing Tat. An enhancing effect of Tat on IL-4 promoter transactivation was also observed after PMA/Io treatment, but was always lower than the effect depending on combined NFAT/AP-1 sites (Fig. 2a).

The above results suggested that Tat affects NFAT/AP-1 cooperativity. To confirm this further, we used another promoter with a composite NFAT/AP-1 site, the human Cox-2 promoter. Again, Tat expression either in the stable transfectants or following transient transfection strongly increased PMA/Io activation of this promoter. More interestingly, the mutation of the NFAT or the AP-1 site strongly diminished the ability of Tat to enhance the transactivation induced by the primary stimulus PMA/Io (Fig. 2b).

Next, we tested whether this effect of Tat was related to its ability to transactivate the LTR. For this, we transfected Jurkat cells with equal amounts of various plasmids encoding Tat mutants. Point mutations that drastically affected this Tat activity did not affect its ability to enhance NFAT/AP-1-dependent transcription in the presence of PMA/Io (Fig. 3). Thus, the Y26A mutation, which was completely inactive in activating HIV-1 LTR transcription (Verhoef & Berkhout, 1999), did not affect the ability of Tat to transactivate NFAT/AP-1 sites.

**Tat acts downstream of nuclear translocation of NFAT**

Next, we tested whether Tat affected the amount of NFAT translocated to the nucleus upon activation using Western blot assays. Nuclear translocation of NFATc2 in response to PMA/Io stimulation seemed to take place with similar kinetics and in similar amounts in both Jurkat-Tat+ and Jurkat-Tat− cells and was inhibited by cyclosporin A (CsA) (Fig. 4). Contamination of the nuclear extract with cytosol

---

**Fig. 2.** Effect of transiently expressed Tat on transcription factor activity and on the Cox-2 promoter in Jurkat cells. (a) Jurkat-Tat− cells transiently transfected with luciferase reporter plasmids as in Fig. 1 were co-transfected with the indicated amount (ng) of pCMV-Tat per 10^6 cells in order to express the Tat viral protein transiently. After transfection, cells were cultured in the absence of stimulation (control) or treated with PMA/Io for 6 h and luciferase activity was evaluated. (b) Jurkat-Tat− and Jurkat-Tat+ or Jurkat cells transiently transfected with the indicated amount (ng) of pCMV-Tat per 10^6 cells, were transiently transfected with the p275-Cox-2-luc plasmid and their NFAT and AP-1 mutated versions. After transfection, cells were cultured in absence of stimulation (used as a control of the fold induction) or treated with PMA/Io (data shown) for 6 h and luciferase activity was evaluated.
was excluded by testing these nuclear extracts by Western blot analysis with antibodies against the cytoplasmic protein marker lactate dehydrogenase (data not shown). In this regard, although the presence of a certain amount of NFATc2 in the nucleus of unstimulated Jurkat-Tat and Jurkat-Tat+ cells is intriguing, similar observations have already been described in Raji and Jurkat cells in which partially dephosphorylated forms of NFATc2 were constitutively found in unstimulated cells (Park et al., 1995).

A suggested explanation is that transformation-associated activation in cell lines may result in nuclear localization of NFAT. Taken together, the above results suggested that the effects of Tat were not mediated by alterations in the calcium-signalling pathway or in NFAT translocation to the nucleus.

**Tat specifically increases cooperative NFAT/AP-1 binding to DNA**

To investigate whether Tat was affecting the binding of the NFAT/AP-1 complex to DNA, we performed EMSAs with the human NFAT/AP-1 oligonucleotide. Activation of Jurkat cells by PMA/Io (Fig. 5a) but not by PMA or Io alone (not shown) led to the formation of a specific complex. CsA inhibited the translocation of NFAT to the nucleus. Interestingly, the amount of complex bound was strongly increased (by ~ fivefold, as determined by densitometric scanning) in Jurkat-Tat+ cells (Fig. 5a). Since this site is a composite element, we decided to investigate whether individual binding was being affected by Tat. Thus, we used oligonucleotides able to bind NFAT (corresponding to the NFAT site of the IL-4 promoter) or AP-1 exclusively in EMSAs. Interestingly, neither of these two sites showed an augmented binding in Jurkat-Tat+ cells when compared with Jurkat-Tat cells, either in basal or in stimulated conditions (Fig. 5b and c).

![Fig. 4.](image1.png) Tat does not affect nuclear NFAT translocation. Jurkat-Tat- and Jurkat-Tat+ cells were stimulated for 0, 10, 45 and 90 min with PMA/Io. The presence of NFATc2 in nuclear extracts (4 μg) was analysed by Western blotting using anti-NFAT1 672 serum.

![Fig. 5.](image2.png) Tat increases the cooperative but not the individual binding of NFAT and AP-1 to DNA. Nuclear extracts of control and stimulated Jurkat-Tat- and Jurkat-Tat+ were analysed by EMSA using probes corresponding to the NFAT/AP-1 distal site of the human IL-2 promoter (a), the P sequence of the murine IL-4 promoter (b), the AP-1 consensus oligonucleotide (c) and the NFAT/AP-1 distal site of the murine IL-2 promoter (d). Stimulation of cells was performed with PMA, Io or PMA/Io for 90 min. CsA (100 ng ml−1) was added 30 min before stimulation as indicated. An 80-fold molar excess of unlabelled homologous oligonucleotide (Comp.) was added to the reaction mixtures to corroborate the specificity of binding. In (d), competition experiments with anti-NFAT and anti-Fos family antibodies are also shown.
The above results suggested that Tat increased the cooperative binding of NFAT and AP-1 to DNA but not their individual binding. To confirm this hypothesis, we took advantage of the binding characteristics of these transcription factors to the NFAT distal site in the murine IL-2 gene promoter, which differs by 2 nt from the human sequence and allows the binding of NFAT even in the absence of AP-1 activation (Macián et al., 2001). Thus, when Jurkat cells were treated with Io, which only activates NFAT, the induced binding of NFAT to the probe was similar in nuclear extracts of stimulated Jurkat-Tat− and Jurkat-Tat+ cells (Fig. 5d). However, when Io was used in combination with PMA (which activates AP-1), NFAT could bind to this probe alone or combined with AP-1, forming two different complexes that migrated differently and could be identified with specific antibodies. Interestingly, in Jurkat-Tat− cells, only the formation of the slower-migrating complex (NFAT/AP-1) was strongly increased (by ~20-fold by densitometric scanning) (Fig. 5d). The most likely explanation is that Tat was interacting with either NFAT or AP-1 (or both), favouring their cooperative binding to these NFAT/AP-1 composite DNA elements.

To check whether similar effects of Tat were observed with NFATc1, another member of the NFAT family present in T lymphocytes, we transfected COS-7 cells with NFATc1 and c-Jun with or without Tat and performed EMSAs with the NFAT/AP-1 composite element of the human IL-2 promoter. COS-7 cells did not express NFAT (Fig. 6, lane 1); a complex with the NFAT/AP-1 probe was formed only when the RSV-c-Jun and pSH107-NFATc1 expression vectors were simultaneously transfected (Fig. 6, lane 2). Simultaneous Tat-HA transfection seemed to increase the amount of complex detected, which ran slightly slower (Fig. 6, lane 4). More interestingly, anti-HA (Tat) antibodies as well as anti-NFAT 676 serum (Fig. 6, lanes 5 and 6, respectively) supershifted the specific complex, whereas anti-c-Jun inhibited it (Fig. 6, lane 7). Addition of a control rabbit serum had no effect on the complexes (not shown). This suggests that a complex between NFAT, c-Jun and Tat may be formed on the DNA probe.

**Tat favours NFAT/AP-1 functional cooperation**

The hypothesis of a direct effect of Tat on the NFAT/AP-1 composite site was reinforced by co-transfection experiments in Jurkat cells. Thus, transfection of Jurkat cells with an NFATc2 expression plasmid led to an ~100-fold increase in the transactivation of the human IL-2 NFAT/AP-1 reporter gene (Fig. 7a). The co-expression of Tat together with NFATc2 increased the observed transcriptional induction further up to ~440-fold over the basal level. Transfection of c-Jun, the main component of AP-1, was able to augment the activity of the reporter ~twofold and also that of NFAT by the same extent (~210-fold over the basal level). Co-transfection of Tat modestly increased c-Jun activity (~twofold). More interestingly, Tat synergistically increased with c-Jun and NFATc2 transactivation up to ~2600-fold over the basal level (or 12-fold over optimal doses of NFAT and c-Jun together) (Fig. 7a). There were no...
significant differences in the amounts of various proteins in transfected cells that could explain these differences (Fig. 7b), thus supporting a strong cooperative effect of Tat with NFAT and c-Jun.

Tat increased NFAT/AP-1 cooperative binding to DNA and favoured their functional cooperation. NFAT–Tat interaction was evidenced by co-immunoprecipitation assays in transfected COS-7 cells. Western blot analysis of the cellular extracts showed that Tat (Fig. 8a, lanes 2 and 3) and NFAT (Fig. 8a, lanes 4 and 5) were detected in COS-7 cells only when transfected with the corresponding expression plasmids. Antibodies against NFATc2 co-precipitated Tat only when both expression plasmids were co-transfected (Fig. 8b, lane 5) and non-specific binding of Tat to anti-NFATc2 was excluded, as no Tat protein was immunoprecipitated in the absence of NFATc2 (Fig. 8b, lanes 2 and 3). Control of Tat and NFATc2 migration in the gels and of the specificity of the antibodies were determined by running total extracts of COS-7 cells transfected with Tat or NFAT (Fig. 8b, lane C). To corroborate this further, we performed pull-down assays of COS-7 cells transfected with either c-Jun or NFATc2. GST–Tat pulled down NFAT but not c-Jun (Fig. 8c).

**DISCUSSION**

HIV-1 Tat affects the activity of several transcription factors and alters the transcription of various cytokines (Cullen, 1998; Stevenson, 2003). However, the underlying mechanisms are unclear. This may stem from the fact that Tat may exert some effects on HIV-1-infected cells as well as on neighbouring bystander cells. Tat may alter cytokine secretion at two levels: (i) by acting in neighbouring cells as a paracrine molecule, and (ii) by affecting transcription in cells infected by HIV-1. In order to investigate how intracellular Tat affects cytokine expression, we analysed its effects on the activity of several of the most important factors controlling cytokine transcription, NFAT, AP-1 and NF-κB (Ghosh *et al.*, 1998; Macián *et al.*, 2001). A previous report showed enhanced transactivation of the NFAT/AP-1 composite element of the human IL-2 gene (Macián & Rao, 1999). Our results are in agreement with these results, but provide additional evidence of the underlying mechanism. Thus, we showed that the expression of HIV-1 Tat in T cells was able to increase strongly NFAT and c-Jun cooperative interactions, resulting in enhanced binding to DNA and transcriptional activity. In the Cox-2 promoter, containing a composite NFAT/AP-1 element (Iñiguez *et al.*, 2000), the enhancing effect of Tat was severely diminished if either the NFAT or AP-1 site was mutated. Signal transduction events that led to either NFAT or AP-1 activation, including transplantation of NFAT to the nucleus, were not affected by intracellular Tat expression, nor did Tat affect the amount of c-Jun and NFAT in these cells.

In Jurkat cells, a strong activation (~210-fold) of the NFAT/AP-1 reporter was observed after overexpressing both NFATc2 and c-Jun. Despite this huge activation, Tat

In Jurkat cells, a strong activation (~210-fold) of the NFAT/AP-1 reporter was observed after overexpressing both NFATc2 and c-Jun. Despite this huge activation, Tat

**Fig. 8.** HIV-1 Tat interacts with NFATc2 but not with c-Jun. (a, b) Co-immunoprecipitation of NFATc2 and Tat. Plasmids pEF-BOS–NFATc2 and/or pCMV-Tat were transfected into COS-7 cells using the indicated amounts of DNA per 5 × 10⁶ cells (~, 0 μg; +, 2.5 μg; ++, 5 μg). Plasmid pcDNA3 was used to adjust the amount of DNA transfected per 5 × 10⁶ cells to 5 μg when necessary. For the detection of NFATc2, 6% polyacrylamide gels were used and for the detection of Tat, 17% polyacrylamide gels were used. In (a), 15 and 25 μl of total cellular extracts (500 μl) were assayed to analyse the expression of NFATc2 and Tat, respectively, by Western blotting. In (b) 200 μl of the total extracts was used for immunoprecipitation with the anti-NFATc2 672 serum. The presence of NFATc2 and Tat in the immune complexes was evaluated by Western blotting. In lane C, an aliquot of the total extracts from COS-7 cells overexpressing NFAT in the top or Tat in the bottom gel was used as an internal control to identify the migration of NFAT and Tat, respectively. (c) GST–Tat pulls down NFATc2 but not c-Jun. COS-7 cells were transfected with the plasmids pEF-BOS–HA–NFATc2 or pCMV–c-Jun–HA and pulled down with GST–Tat and blotted with an anti-HA antibody. Cell extracts of the input samples are also shown.
increased this transactivation further (up to ~2600-fold). Taken together, these results suggest a mechanism involving direct interactions between Tat and the NFAT/AP-1 complex, which take place only at these composite elements. This hypothesis was supported further by: (i) the enhanced binding of the NFAT/AP-1 complex to the composite element in the IL-2 promoter observed in Jurkat-Tat cells compared with wild-type cells or after the addition of recombinant Tat to the nuclear extracts of Jurkat wild-type cells and in COS-7 cells overexpressing NFAT, c-Jun and Tat; (ii) the ability of anti-Tat antibodies to supershift or to prevent complexes bound to the NFAT/AP-1 DNA probe in COS-7 cells overexpressing NFAT, c-Jun and Tat. Moreover, the existence of a direct interaction between NFAT and Tat was demonstrated by co-immunoprecipitation assays in cellular extracts of COS-7 cells when both proteins were co-expressed. These data confirm those obtained previously by Macián & Rao (1999) using cellular extracts of HEK293 cells co-transfected with both expression plasmids. In addition, we found that GST–Tat pulled down NFAT but not c-Jun from transfected COS-7 cells. Although the direct NFAT–Tat interaction leads to increased transactivation in Gal4–NFAT assays (Macián & Rao, 1999), this could not explain satisfactorily the increased transactivation with the NFAT/AP-1 reporter plasmid, as these effects were less pronounced than those observed with the combined NFAT/AP-1 site. Moreover, this single interaction with NFAT was not sufficient to explain the increased binding of the NFAT/AP-1 complex to this composite element, as Tat does not affect the binding of NFAT to sequences where this transcription factor binds independently from AP-1. Therefore, we concluded that the NFAT–Tat interaction strongly potentiates its cooperation with AP-1.

Binding of AP-1 to the NFAT/AP-1 distal site of the human IL-2 gene promoter is strongly dependent on its interaction with NFAT. For NFATc1, this interaction takes place through amino acids near the N-terminal region (Peterson et al., 1996) where the interaction between NFATc2 and Tat has been localized (Macián & Rao, 1999). Therefore, we believe that Tat interaction with NFAT may affect NFAT–AP-1 contacts and, subsequently, binding of the complex to DNA. In addition, the NFAT–AP-1 interaction at this composite element differs from other protein–protein interactions by its marked electrostatic character (Sun et al., 1997) and Tat has strongly charged regions (Truant & Cullen, 1999). Consequently, alteration of electrostatic interactions in the NFAT/AP-1 complex as a result of the binding of Tat to NFAT could be a possible mechanism to explain the observed effects.

We did not detect alterations by transient or stable expression of Tat either in the binding or in the transactivation activities of AP-1. HIV-1 Tat was not able to interact physically with c-Jun, nor to potentiate its transactivating activity (not shown) or affect AP-1 binding. In spite of this, Tat may interact with AP-1 through interaction with other transcription factors. In this regard, Lim & Garzino-Demo (2000) found that Tat interaction with Sp1 at the human MCP-1 gene promoter may serve as a platform to recruit and stabilize the interaction of AP-1 and NF-κB proteins to this promoter. In addition, HIV-1 Tat can activate the Jun kinase enzyme (JNK), which is required for AP-1 activation (Biswa et al., 1995; Li et al., 1997; Kumar et al., 1998). However, these effects were observed with extracellular Tat and Tat-neutralizing antibodies inhibited JNK activation. In our experiments, Tat secretion by Jurkat-Tat cells could be insufficient to achieve the extracellular concentration required to act on neighbouring cells and affect JNK activation.

The selective effect of Tat on DNA target sequences where NFAT and AP-1 bind cooperatively may have important functional implications, as many genes (e.g. IL-2, Cox-2, IL-4, IL-5, CD40L, GM-CSF) are regulated by NFAT/AP-1 composite elements with defined structural requirements (Kel et al., 1999; Macián et al., 2001). The distal NFAT/AP-1 site of the human IL-2 promoter is a representative example of nuclear factor cooperation; NFAT increases the affinity of AP-1 10-fold (Peterson et al., 1996) and the NFAT/AP-1/DNA complex is 10 times more stable than the NFAT/DNA complex (Jain et al., 1993). In this way, the cooperative binding of both transcription factors allows the complete function of this response element. The effect of Tat on the cooperative interactions between such transcription factors and DNA may represent a tuned HIV-1 strategy to regulate the expression of certain genes depending on those composite elements that are critically involved in the immune response, such as IL-2 and the above-mentioned proteins. In addition, our results show that these effects take place at very low levels of expression of the viral protein in the cell, close to the physiological situation. Interestingly, these effects of Tat do not seem to be related to its ability to transactivate the LTR. Thus, theoretically HIV-1 strains partly defective in active virus replication could still modulate NFAT/AP-1 transcription and produce cytokine dysregulation.

Moreover, aside from the impact shown at the cellular level, the described mechanism may play a feedback role in the level of viral transcription in a subtype-specific manner. Indeed, in the HIV-1 LTR, enhancer NFAT (NF-κB) and AP-1 binding sites are represented and spaced differently, depending on the viral subtype being considered (Jeeninga et al., 2000; van Oprijen et al., 2004; Centlivre et al., 2005). Thus, the enhancement induced by Tat on the cooperation between NFAT and AP-1 might be relevant for some major subtypes (e.g. A and C) and irrelevant for others (e.g. B).

Moreover, NFAT is rarely able to bind alone to DNA. Rather, it usually forms cooperative interactions with other factors besides AP-1 (Hogan et al., 2003; Macián et al., 2001). Thus, interactions with C/EBP in the peroxisome proliferator activated receptor and insulin-like growth factor promoters (Yang & Chow, 2003) or with IRF-8 in the IL-12 promoter (Zhu et al., 2003) have been described. Tat may modulate (potentiate or inhibit) these interactions by interacting with NFAT. Experiments are in progress to
Tat protein induces CD69 expression through NF-kappaB activation:

J. M., Thomas, D., Diaz-Meco, M. T., Moscat, J. & Virelizier, J. L.

Arenzana-Seisdedos, F., Fernandez, B., Dominguez, I., Jacque,
transcriptional regulator of mammalian cell cycle.

Physical and functional interaction of HIV-1 Tat with E2F-4, a
biding protein to the IL6 leader RNA and by interacting with CAAT enhancer-

induces the expression of the interleukin-6 (IL6) gene by binding

HIV-1 Tat modulates MCP-1 gene transcription in astrocytes.

In vitro and in vivo binding of human immunodeficiency virus type 1 Tat protein and Sp1 transcription factor.

an essential role of the nuclear factor of activated B and Rel proteins:


An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes.

J Immunol 159, 837–848.

Jeeninga, R. E., Hoogenkamp, M., Armand-Ugon, M., de Baar, M.,

ACKNOWLEDGEMENTS

We thank Dr J. Alcami and Dr J. M. Redondo and his group for helpful
discussions and for kindly providing us with reagents that made this
work possible. We are also grateful to those who have helped us with
different reagents as mentioned in Methods and to Maria Chorro,
Lucia Horrillo and Gloria Escribano for their excellent technical
assistance. This work was supported by grants from the Ministerio de
Educación y Ciencia-FEDER (SAF 2004-05109), RECAVA cardio-
vascular network (603/01) and RIS (603/173) from Fondo de
Investigaciones Sanitarias; EICOSANOX integrated project and
MAIN network of excellence from the 6th EU Framework Pro-
gramme; Laboratorios del Dr ESTEVE; Comunidad de Madrid (08.3/ 0007/1), Fundación para la Investigación y Prevención del SIDA en España (FIPSE) and Fundación Ramón Areces. A. M. H.-E. is a holder of an FPU fellowship of the Ministerio de Educación y Ciencia.

REFERENCES

Cooperative interaction of C/EBPβ and Tat modulates MCP-1 gene transcription in astrocytes.

Ambrosino, C., Ruocco, M. R., Chen, X., Mallardo, M., Baudì, F.,
HIV-1 Tat induces the expression of the interleukin-6 (IL6) gene by binding to the IL6 leader RNA and by interacting with CAAT enhancer-binding protein β (NF-IL6) transcription factors.

Ambrosino, C., Palmieri, C., Puca, A. & 9 other authors (2002).
Physical and functional interaction of HIV-1 Tat with E2F-4, a transcriptional regulator of mammalian cell cycle.

Arenzana-Seisdedos, F., Fernandez, B., Domínguez, I., Jacque,
J. M. Thomas, D., Diaz-Meco, M. T., Moscat, J. & Virelizier, J. L.

Differential expression of transcription directed by a discrete NF-AT binding element from the IL-4 promoter in naive and effector CD4 T cells.
J Immunol 159, 36–43.

A Tat-induced auto-up-regulatory loop for superactivation of the human immunodeficiency virus type 1 promoter.

Blazquez, M. V., Macho, A., Ortiz, C., Lucena, C., Lopez-Cabrera, M.,
Extracellular HIV type 1 Tat protein induces CD69 expression through NF-kappaB activation: possible correlation with cell surface Tat-binding proteins.
AIDS Res Hum Retroviruses 15, 1209–1218.

Buonaguro, L., Barillari, G., Chang, H. K., Bohan, C. A., Kao, V.,
Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines.

Centlivre, M., Sommer, P., Michel, M. & 8 other authors (2005).
HIV-1 clade promoters strongly influence spatial and temporal dynamics of viral replication in vivo.

HIV-1 auxiliary proteins: making connections in a dying cell.
Cell 93, 685–692.

Human immunodeficiency virus type 1 Tat protein activates transcription factor NF-xB through the cellular interferon-inducible, double-stranded RNA-dependent protein kinase, PKR.

JunB differs from c-Jun in its DNA-binding and dimerization domains and represses c-Jun by formation of inactive heterodimers.
Genes Dev 7, 479–490.

Characterization of antigen receptor response elements within the interleukin-2 enhancer.

Cellular uptake of the tat protein from human immunodeficiency virus.
Cell 55, 1189–1193.

NF-xB and Rel proteins: evolutionarily conserved mediators of immune responses.

HIV-1 Tat inhibits IL-2 gene transcription through qualitative and quantitative alterations of the cooperative Rel/AP1 complex bound to the CD28RE/API complex element of the IL-2 promoter.
J Immunol 166, 4560–4569.

Transactivation of human immunodeficiency virus gene expression is mediated by nuclear events.
Proc Natl Acad Sci U S A 84, 6364–6368.

Transcriptional regulation by calcium, calcineurin and NFAT.
Genes Dev 17, 2217–2232.

Activation and deactivation of gene expression by Ca2+/calcineurin-NFAT-mediated signaling.
Mol Cells 18, 1–9.

An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes.

Izmailova, E., Bettle, F. M. N., Huang, Q., Makori, N., Miller, C. J.,
HIV-1 Tat reprograms immature dendritic cells to express chemokinreceptors for activated T cells and macrophages.
Nat Med 9, 191–197.

J Immunol 151, 837–848.

Jeeninga, R. E., Hoogenkamp, M., Armand-Ugon, M., de Baar, M.,
Functional differences between
the long terminal repeat transcriptional promoters of human immunodeficiency virus type 1 subtypes A through G. J Virol 74, 3740–3751.


