Extracellular HIV-1 Tat Protein Induces the Rapid Ser \textsuperscript{133} Phosphorylation and Activation of CREB Transcription Factor in Both Jurkat Lymphoblastoid T Cells and Primary Peripheral Blood Mononuclear Cells

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Extracellular HIV-1 Tat Protein Induces the Rapid Ser\textsuperscript{133} Phosphorylation and Activation of CREB Transcription Factor in Both Jurkat Lymphoblastoid T Cells and Primary Peripheral Blood Mononuclear Cells\textsuperscript{1}

Davide Gibellini,*, Alessandra Bassini,† Sabina Pierpaoli,† Lucia Bertolaso,† Daniela Milani,† Silvano Capitani,† Michele La Placa,* and Giorgio Zauli\textsuperscript{2†}

Extracellular HIV-1 Tat protein (0.1–100 ng/ml) induced a rapid (peak at 30 min) increase in the Ser\textsuperscript{133} phosphorylation levels of the transcription factor CREB in serum-starved Jurkat cells, as revealed by Western blot and indirect immunofluorescence analyses. Nuclear cAMP-responsive element (CRE) binding activity in electrophoretic mobility shift assays was constitutive in unstimulated Jurkat cells, showing only a small increase upon Tat treatment. However, transient transfection experiments performed with various chloramphenicol acetyl-transferase (CAT) constructs showed that Tat produced a fourfold induction of CAT activity only in the presence of a CRE-dependent CAT construct. Moreover, the use of plasmids encoding for GAL4-CREB fusion proteins demonstrated that Tat induction of pG4-CAT reporter gene required the CRE moiety of the GAL4-CREB fusion protein and that Ser\textsuperscript{133} CREB was essential for Tat activity. Extracellular Tat also stimulated Ser\textsuperscript{133} CREB phosphorylation in freshly isolated PBMC; this effect was completely blocked by either staurosporin, a broad-spectrum inhibitor of various protein kinases, or PD 98059, a specific inhibitor of mitogen-activated protein kinases (MAPK). Furthermore, extracellular Tat induced a rapid (peak at 5–15 min) stimulation of the MAPK catalytic activity in primary PBMC. Altogether, these findings suggest that HIV-1 Tat protein activates CREB in lymphoid cells through a signal cascade involving the MAPK pathway. The Journal of Immunology, 1998, 160: 3891–3898.

The regulatory Tat protein of HIV-1 is essential for an efficient viral replication (1). The coding sequences of the tat gene are located in two exons, one within the central region of the viral genome and a second that overlaps the envelope gene. The spliced tat mRNA is translated into an 86 to 101 amino acid Tat protein, which transactivates HIV-1 gene expression by acting mainly on a stable RNA hairpin (the transactivation response element or TAR) that forms the 5′-untranslated leader of nascent viral transcripts (2–3). Tat can be divided into five distinct domains: N-terminal, cysteine-rich, core, basic, and C-terminal sequences. The cysteine-rich region is responsible for the formation of intramolecular disulfide bonds (4), while the basic region contains nuclear localization signals (5) and the binding site to TAR. The activation domain of Tat (amino acids 1–48) includes the amino-terminal region and the conserved cysteine-rich and core domains (1).

Data from different groups of investigators indicate that Tat protein can be actively secreted by either HIV-1-infected or tat-transfected cells (6–9), and it displays pleiotropic activities on cell survival, growth, and function of bystander uninfected cells (6–17). In particular, it has been demonstrated that high (100 nM-10 \( \mu \)M) concentrations of extracellular Tat show a cytotoxic effect on the proliferation of isolated T lymphocytes driven by recall Ags (11–14) as well as on Jurkat cells (15–17). On the other hand, lower (10 pM-1 nM) concentrations of Tat protein promote the survival/growth of different cell types, including primary PBMC and Jurkat cells (18).

Besides being released by HIV-1-infected cells, Tat protein can be taken up by different cell types both in vitro (19–21) and in vivo (22), reaching the nucleus quite rapidly. Therefore, it has been proposed that the biologic effects of high Tat concentrations on T cell survival/growth are mainly due to a direct action of Tat at the nuclear level on the promoters of cellular genes encoding for cytokines (23–27) or genes relevant to cell survival or transformation (14, 18, 28, 29). However, it has also been shown that Tat, at low concentrations, displays a cytokine-like activity, able to interact with a variety of surface receptors (30–35) and to activate phosphatidylinositol 3-kinase (PI-3)\textsuperscript{3} (36) and protein kinase C (PKC) (37–38). The cAMP-responsive element binding protein (CREB) transcription factor has been identified as a substrate for several kinases (39–41). While the role of progressive phosphorylation by casein kinase I and II at other serine residues remains undefined, it is well established that phosphorylation of CREB at Ser\textsuperscript{133} is essential for gene transactivation mediated by CRE sites placed in the promoters of many cellular or viral genes (42–46). CREB

\textsuperscript{1} Institute of Microbiology, University of Bologna, Bologna, and \textsuperscript{2} Institute of Human Anatomy, University of Ferrara, Ferrara, Italy

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\textsuperscript{3} Abbreviations used in this paper: PI-3, phosphatidylinositol-3 kinase; PKC, protein kinase C; PKA, protein kinase A; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; CAT, chloramphenicol acetyl-transferase; GAR, goat anti-rabbit; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinase.
plays a key role in the physiology of nervous and neuroendocrine systems (40, 45), whereas its potential relevance in the regulation of lymphoid cell survival/growth still remains to be clearly defined. In this respect, it has been recently demonstrated that CREB might be implicated also in T cell development (47) and proliferation (48).

To investigate the ability of Tat to activate intracellular signals in lymphoid cells, we evaluated here the effect of Tat on CREB phosphorylation levels, CRE-binding activity in nuclear extracts, and CRE-CAT activity in lymphoblastoid Jurkat T cell line. In parallel experiments, extracellular Tat was added to primary PBMC in the absence or presence of a variety of pharmacologic inhibitors of different signal transduction pathways.

Materials and Methods

**Reagents**

Synthetic (Technogen, Caserta, Italy) HIV-1 Tat and recombinant HIV-1 p24 (Intracell, Cambridge, MA) proteins were dissolved in PBS containing 0.1% BSA and aliquoted at −70°C before use. Staurosporin, wortmannin, bisindolylmaleimide, chelerythrine, H-89, PD 98059, and forskolin, all purchased from Calbiochem (La Jolla, CA), were prepared in DMSO and stored at −20°C.

**Cell lines, PBMC, and treatment of cells**

Human Jurkat CD4+ lymphoblastoid T cell line was maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Life Technologies) at an optimal cell density of 0.3 to 1.5 × 10^6 cells/ml. PBMC were obtained from normal blood donors, who gave their informed consent to this research according to the Helsinki Declaration of 1975. Briefly, heparinized (20 U/ml) blood samples were diluted 1:3 with RPMI plus 10% FCS, layered over Ficoll Histopaque (density = 1.077 g/ml; Pharmacia, Uppsala, Sweden) and centrifuged at 1500 rpm for 30 min. Light density mononuclear cells were collected, washed twice, counted, and seeded in culture in RPMI plus 0.1% BSA.

In most experiments, 48-h serum-starved (RPMI + 0.1% BSA) Jurkat cells or freshly isolated PBMC were seeded in culture in the absence or presence of increasing (0.1–1000 ng/ml) concentrations of Tat protein for 0 to 90 min. HIV-1 p24 was used as negative control.

In blocking experiments performed with the pharmacologic inhibitors staurosporin, wortmannin, bisindolylmaleimide, chelerythrine, and PD 98059, cells were pretreated for 1 h at 37°C with various doses of each inhibitor diluted in RPMI medium, while control cells were treated with DMSO diluted in RPMI.

**Western blotting and indirect immunofluorescence staining revealed by flow cytometry or confocal microscopy**

Samples derived from 2 to 10^6 viable Jurkat cells or PBMC, containing approximately 100 μg of proteins, were migrated on polyacrylamide gels and blotted onto nitrocellulose filters. Blotted filters were blocked for 30 min in a 3% suspension of dried skimmed milk in PBS, then incubated overnight at 4°C with 1:1000 dilution of anti-CREB serum in PBS or serum directed against the phosphorylated Ser^133^ form of CREB (both from Upstate Biotechnology, Lake Placid, NY). Anti-Ser^133^ CREB serum was revealed by flow cytometry or confocal microscopy.

For confocal microscopy, the cells were first spun on coverslips, then fixed and stained with the anti-Ser^133^ CREB serum as described above. The cells were analyzed by means of a MCR-1000 confocal microscope (Bio-Rad Microscience, Hemel Hempstead, U.K.) equipped with a krypton/argon ion laser emitting at 488 nm. The signal was achieved through an Epitope filter (passing band 522/35 nm), analyzed by CoMOS software and printed on a Ektachrome 64T Kodak film by a Focus Imagecorder Plus (Focus Graphics, Foster City, CA).

**Electrophoretic mobility shift assay (EMSA)**

A CRE site (5'-AGAGATTGCTGTCACAGAGACGTA-3'), containing double-stranded oligonucleotide (Promega, Madison, WI), was 5'-labeled with [α-32P]ATP, purified, and used as a probe in EMSAs. The underlined portion of the preceding sequence is the CRE site. Briefly, nuclear protein extracts (4–8 μg protein) prepared from Jurkat cells by the method of Dignam et al. (49) were incubated with 3 μg of poly(dI-dC)-dC) and 20,000 cpm of [32P] end-labeled oligonucleotide probe for 20 min at room temperature in a 20-μl solution containing 15% glycerol, 20 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 5 mM MgCl2, 1 mM EDTA, and 1 mM DTT. For supershift analysis, 5 μg of anti-CREB sera or of normal rabbit serum were added to reaction mixtures 5 min before the addition of labeled CRE-containing oligonucleotide. Protein-DNA complexes and protein-DNA-ssupershift complexes were resolved in 4% polyacrylamide gels in 0.1× TBE buffer (22.5 mM Tris-borate and 0.5 mM EDTA, pH 8.3). Gels were dried and exposed overnight to radiographic film with an intensifying screen at −70°C.

**Plasmid and transfection experiments**

The following plasmids were a generous gift of Dr. Enzo Lalli (IGBMC, Strasbourg, France): 1) CRE-CAT (pSom-CAT) plasmid (50), which represents the CRE motif of the rat somatostatin promoter cloned in front to the chloramphenicol acetyl-transferase (CAT) gene in pBLCAT2 vector; 2) pBLCAT2 backbone vector; 3) pGAL4 encoding for amino acids 1 to 147 of GAL4 yeast protein (DNA binding domain, GAL4_147); 4) pGAL4-CREBΔLZ, which lacks the COOH-terminal 29 amino acids of GAL4-CREB, including the leucine repeat dimerization motif (LZ); 5) pGAL4-CREBΔLZM1, representing the pGAL4-CREBΔLZ with the Ser^133^ to Ala mutation (51); and 6) pG4-CAT (52), containing 4× GAL4 sites cloned in front of herpes simplex virus thymidine kinase promoter.

Transient transfection experiments were performed using the DEAE-dextran method, as described previously (18). In single transfection experiments, 10^7 Jurkat cells were maintained in RPMI 1640 plus 1% FCS for 24 h and then transfected with 10 μg of CRE-CAT or pBLCAT2 in 500 μg DEAE-dextran for 90 min. Twelve hours after transfection, cells were treated with 10 ng/ml of Tat, 10 ng/ml of p24, or 10 μM forskolin or left untreated. In some experiments, Tat was pretreated with 1 μg of anti-Tat-neutralizing mAb (Intracell) for 1 h at 37°C before adding to the cells. Twenty-four hours after treatment with Tat and other agonists, Jurkat cells were lysed and assayed for CAT activity using volumes of extract corresponding to equal protein amounts. Protein determination was performed by Bradford assay (Bio-Rad, Richmond, CA).

In cotransfection experiments, 10^7 Jurkat cells were transfected with 10 μg of pG4CAT plus pGAL4 or pGAL4-CREBΔLZ or pGAL4-CREBΔLZM1 in 500 μg DEAE-dextran for 90 min. Twelve hours after transfection, cells were treated with the same agonists described above for 24 h and assayed for CAT activity.

**Mitogen-activated protein kinase (MAPK) assay**

In some experiments, the kinase catalytic activity of MAPK was analyzed in whole homogenates obtained from serum-starved Jurkat cells or freshly isolated primary PBMC treated for different time points with 10 ng/ml of extracellular Tat or p24. For this purpose, the Biotrak kit p42/p44 MAPK enzyme assay system was used, according to the manufacturer’s instructions (Amersham). The data obtained were expressed in pmol of phosphate (P) per min.

**Statistical analysis**

Statistical analysis was performed using Student’s two-tailed t test.
of Ser\textsuperscript{133} CREB phosphorylation, which reached the maximal level in the total amount of CREB were noticed upon Tat treatment (Fig. 1A).

On the other hand, no significant modifications in the duration of high-affinity surface receptors with occupancy of low-affinity receptors by Ensoli et al. (7). This phenomenon might reflect the saturation of endogenous Ser\textsuperscript{133}-phosphorylated CREB in cells cultured in RPMI plus 10% FCS. Forty-eight hours after serum starvation, Jurkat cells were stimulated with increasing concentrations of extracellular synthetic Tat protein for 30 min. As positive control of CREB Ser\textsuperscript{133} phosphorylation, we used forskolin (10 \muM), a well-known activator of protein kinase A (PKA). Cell homogenates were next analyzed by Western blot analysis with both anti-Ser\textsuperscript{133} phosphorylated CREB (Fig. 1A) and anti-CREB (Fig. 1B) sera.

While the CREB phosphorylation levels were very weak or undetectable in serum-starved Jurkat cells, 0.1 to 100 ng/ml of Tat protein induced a significant increase in a dominant, inducible protein of relative molecular size 43 to 46 kDa (Fig. 1A). Maximal stimulation was achieved at 10 ng/ml, while 1000 ng/ml of Tat failed to significantly stimulate CREB phosphorylation. A similar bell-shaped response to increasing concentrations of extracellular Tat has been previously reported from a study of Kaposi's sarcoma cells by Ensoli et al. (7). This phenomenon might reflect the saturation of high-affinity surface receptors with occupancy of low-affinity receptors. On the other hand, no significant modifications in the total amount of CREB were noticed upon Tat treatment (Fig. 1B). The changes in intensity of the immunoreactive bands were quantified by densitometry and expressed in arbitrary units.

A time course of Tat-mediated CREB phosphorylation was next examined in Jurkat cells (Fig. 2, A and B) and primary PBMC (Fig. 2, C and D). Extracellular Tat (10 ng/ml) induced a rapid increase of Ser\textsuperscript{133} CREB phosphorylation, which reached the maximal level after 30 to 60 min in both Jurkat and PBMC and showed a decline toward basal levels after 90 min (Fig. 2, A and C). Once again, no significant variations in total CREB levels were noticed using an anti-CREB serum (Fig. 2, B and D). In this and other experiments, instead of a single band, a doublet of immunoreactive proteins was sometimes observed. The faster migrating band may represent a different CREB isoform or a degradation product. As stripping and reprobing with the anti-CREB serum did not allow for discrimination between these two possibilities, due to the proximity of the two phosphorylated bands, densitometric analysis was calculated considering an area that comprised both phosphoproteins.

The Tat-mediated induction of Ser\textsuperscript{133} CREB phosphorylation was also confirmed in Jurkat cells by indirect immunofluorescence staining, revealed by flow cytometry (Fig. 3, A–C) or confocal microscopy (Fig. 3, D–F).

### Results

**Tat protein induces the rapid Ser\textsuperscript{133} phosphorylation of CREB in both Jurkat cells and primary PBMC**

Since previous data from our group and others clearly indicate that extracellular HIV-1 Tat protein can act as a viral growth factor (6–9), in the first group of current experiments we investigated whether Tat was able to modulate the phosphorylation levels of the transcription factor CREB. To do so, Jurkat cells were serum starved (RPMI + 0.1% FCS) to lower the high level of endogenous Ser\textsuperscript{133}-phosphorylated CREB in cells cultured in RPMI plus 10% FCS. Since previous data from our group and others clearly indicate that extracellular synthetic Tat for various amounts of time. Cells were treated with 10 \muM forskolin for 30 min (positive control, lane 1); and 10 ng/ml of Tat for 0 (lane 2), 5 (lane 3), 15 (lane 4), 30 (lane 5), 60 (lane 6), and 90 (lane 7) min. A representative of five separate experiments is shown. The densitometric analysis is expressed in arbitrary units (a.u.).

**Tat enhances the formation of CRE-protein complexes containing Ser\textsuperscript{133} phosphorylated CREB and activates the CRE-CAT reporter gene in Jurkat cells**

Further studies were undertaken to examine whether extracellular Tat had some effect on the nuclear CRE complexes in Jurkat cells. For this purpose, a DNA-binding assay was performed in the absence or presence of sera to CREB, Ser\textsuperscript{133} phosphorylated CREB, or normal rabbit serum. As shown in Figure 4A, nuclear extracts from Jurkat cells typically produced a single dominant nucleoprotein complex. It was shown that this complex contained CREB by complete immunoinhibition with anti-CREB serum (lanes 5 and 8 of Fig. 4A). By using a different, supershifting serum that specifically recognizes only the phosphorylated form of Ser\textsuperscript{133} CREB (lanes 4 and 7 of Fig. 4A), we probed the presence of Ser\textsuperscript{133} CREB after Tat stimulation. On the other hand, no supershift was observed when normal rabbit serum was used (lane 3 of Fig. 4A). The presence of two supershifted complexes in our assay was probably due to the ability of CREB to heterodimerize with various transcription factors, including ATF and c-Fos, that give rise to nuclear protein complexes of different molecular weights (40). The specificity of the band was also demonstrated by a competition study.
analysis of CRE-binding activity by excess cold CRE oligonucleotides (Fig. 4B).

In the next experiments, we evaluated the ability of Tat protein to stimulate CRE-CAT expression in Jurkat cells. Transient transfections were performed using the DEAE/dextran method, which is an efficient system for rapidly screening the effects of heterologous genes on host cell physiology (53). Tat (10 ng/ml) and forskolin (10 μM), which induced phosphorylation of CREB Ser 133, also induced a significant (p < 0.05) increase of CRE-dependent gene expression in Jurkat cells, whereas p24, which does not induce CREB phosphorylation, failed to induce transactivation, fully in keeping with the prevailing view of CREB activation (Fig. 5). Moreover, pretreating Tat protein with an anti-Tat-neutralizing mAb resulted in an almost complete blocking of Tat-mediated CRE-CAT activation.

**Tat inducibility of reporter genes with GAL4-CREB constructs**

The ability of CREB to contribute to Tat signaling was further investigated using a gene that encodes a chimeric protein in which CREB is fused at its amino terminus to the DNA-binding and dimerization domain of the yeast transcription factor, GAL41–147. We employed a specific GAL4-CREB mutant (GAL4-CREBΔLZ) in which the CREB leucine zipper region was deleted, while the GAL4 DNA-binding and dimerization domains remained intact. This mutant was used to rule out possible interactions of full length CREB with cellular proteins containing leucine zipper regions. To determine the functional relevance of CREB Ser133 phosphorylation, we also conducted transfection experiments using GAL4-CREBΔLZM1 construct in which Ser133 was mutated to Ala. It has been shown previously that this specific mutation does not affect the stability of CREB protein or its nuclear localization (51). All cotransfection experiments were performed using a CAT reporter plasmid (pG4-CAT) containing four GAL4 binding sites (Fig. 6).

In the presence of GAL4-CREBΔLZ, CAT reporter gene expression showed a threefold increase in induction levels of Jurkat cells upon treatment with extracellular Tat (10 ng/ml) and a fivefold increase upon treatment with forskolin. In the presence of GAL4-CREBΔLZM1 mutant, Tat did not induce a significant activation of CAT expression. Moreover, Tat failed to induce transcription of the reporter gene if cells were transfected with only GAL41–147. Thus, Tat induction of pG4-CAT reporter gene required the CREB moiety of the GAL4-CREBΔLZ fusion protein.
induced some suppression of Ser133 CREB phosphorylation only when PKA inhibitors were used (data not shown). Of note, wortmannin did not reproducibly affect phosphorylation, which is in line with what was previously described (10). Three separate experiments is shown.

A

CREB

B

CRE/ nuclear protein complex

1 2 3 4 5 6 7 8

FIGURE 4. EMSA using a 32P-labeled CRE site containing oligonucleotides. A, Nuclear extracts were derived from Jurkat cells treated with 10 μM forskolin (lane 1), 10 ng/ml of Tat (lanes 2–5), or left untreated (lanes 6–8). Supershift was performed by incubating nuclear extracts with 5 μl of normal rabbit serum (lane 3), anti-Ser133 CREB serum (lanes 4 and 7), or anti-CREB serum (lanes 5 and 8) for 15 min before adding radiolabeled oligonucleotide probe. The position of the supershifted complexes is indicated by asterisks. B, Nuclear extracts derived from Jurkat cells treated with 10 ng/ml of Tat were preincubated without (lane 1) or with 2 (lane 2), 10 (lane 3), and 50 (lane 4) μM molar excess of unlabeled oligonucleotides for 15 min before adding radiolabeled oligonucleotide probe. Electrophoresis was extended to provide clear separation of supershifted bands; oligonucleotide probe alone is not visualized for this region. A representative of three separate experiments is shown.

Tat stimulates CREB Ser133 phosphorylation through a MAPK-dependent pathway

In the next experiments, we sought to investigate whether Tat-stimulated CREB Ser133 phosphorylation via an intracellular signal cascade. For this purpose, we used different concentrations of the broad-spectrum protein kinase inhibitor staurosporin (50–500 nM), or the more selective PKC inhibitors bisindolylmaleimide (100–1000 nM) and chelerythrine (10–100 μM), the PKA inhibitor H-89 (100–1000 nM), and the PI-3 kinase inhibitor wortmannin (100–1000 nM).

Western blot analysis of primary PBMC homogenates showed that only staurosporin and wortmannin affected the levels of Tat-stimulated Ser133 CREB phosphorylation (Fig. 7 A and B), while no reproducible variations were noticed when selective PKC or PKA inhibitors were used (data not shown). Of note, wortmannin induced some suppression of Ser133 CREB phosphorylation only at concentrations of 500 to 1000 nM, which are significantly higher than those required to obtain a maximal inhibition of PI-3 kinase (100 nM) (54), but are in the optimal range to inhibit the MAPK pathway. Therefore, PBMC were treated next with 10 to 50 μM of PD 98059, a specific inhibitor of MAPK kinase, the upstream enzyme involved in MAPK activation (Fig. 7, C and D). At both concentrations, PD 98059 was specifically able to inhibit the Tat-mediated Ser133 phosphorylation of CREB.

The direct proof that Tat was able to stimulate the catalytic activity of MAPK was obtained in the last group of experiments, in which freshly isolated PBMC were treated with 10 ng/ml of Tat or p24 proteins for different lengths of time. As shown in Figure 8, Tat induced a significant (p < 0.05) increase of MAPK catalytic activity, reaching a peak after 5 to 15 min from the beginning of the treatment. Similar findings were observed using Jurkat cells instead of PBMC (data not shown).

Discussion

The CRE consensus sequence is recognized by several proteins that are members of the CRE-binding transcription factor family, including the activating transcription factors (ATF) subfamily, CREB, CRE-BP1, and others (44–45). All members of the CREB/ATF family have a carboxyl terminus containing a leucine zipper dimerization domain juxtaposed with a DNA-binding domain rich in basic amino acids. This complex superfAMILY of transcriptional transactivator proteins specifically bind to the CRE motif 5′-TGACGTCA-3′ (44).

The specific DNA promoter/enhancer element located in the control regions of the genes serves as target sites for the binding of transcriptional transactivator proteins. The most thoroughly studied CRE-binding protein is a 43- to 46-kDa protein termed CREB, which is expressed constitutively. In fact, CREB activation results from posttranslational modifications such as phosphorylation of Ser133 in situ. This phosphorylation step is critical for transactivation of a CRE-dependent promoter.

Extensive studies have established the role of CREB in mediating transcriptional activation induced by cAMP via a direct phosphorylation of CREB by PKA on Ser133 (39–45). In particular, in the well-known model of PC12 rat pheochromocytoma cells, phosphorylation of CREB Ser133 occurs in response to pharmacologic...
agents that increase intracellular levels of cAMP, acting through PKA, but also in response to nerve growth factor, acting through the Ras/Raf/MAPK pathway (41). In addition to the PC12 cell model, it has been demonstrated also in hemopoietic cells that CREB appears downstream of a variety of intracellular signal transduction pathways that activate receptor tyrosine kinases (55). In fact, the kinase-inducible domain of the CREB gene contains consensus phosphorylation sites for a variety of protein kinases, including PKA, PKC, casein kinases I and II, and calcium-calmodulin-dependent protein kinases I and II (39, 40). Moreover, a specific CREB kinase has been recently identified downstream of the Ras/Raf/MAPK pathway (56).

In this study, we have demonstrated that HIV-1 Tat protein induces the rapid Ser\textsuperscript{133} phosphorylation of CREB in both the Jurkat cell line and primary PBMC. The binding to DNA CRE sites and the increased functional activity of phosphorylated CREB in Tat-stimulated Jurkat cells was then demonstrated by supershift EMSA assay and transient transfection experiments, respectively.

Although the potential physiologic significance of CREB in regulating lymphoid cell survival/growth remains to be defined, our findings may contribute to explaining the previously reported ability of extracellular Tat to modulate CD4\textsuperscript{+} T cell growth (9-17). In fact, it has been recently demonstrated that transgenic mice expressing a dominant-negative form of CREB show defective thymocyte proliferation and IL-2 production (47) and CREB/ATF regulate the cell cycle-controlled proliferating cell nuclear Ag, PCNA (48). Remarkably, a recent study reported that the U5 region of the HIV-1 long terminal repeat contains Tat-responsive element (TRE)-like cAMP-responsive elements binding both AP-1 and CREB/ATF proteins (57), which suggests that enhanced CREB expression is beneficial for HIV-1 replication.

As we have previously demonstrated that extracellular Tat rapidly stimulates PI-3 kinase (36) and PKC (37–38), blocking experiments were performed using various pharmacologic inhibitors. The failure also of the PKC inhibitors bisindolylmaleimide (100–1000 nM) and chelerythrine (10–100 μM) to show any inhibition at the highest concentrations used indicated that PKC is presumably not involved in the Tat-mediated induction of CREB phosphorylation. Also, the PKA inhibitor H-89 did not show any inhibitory effect. On the other hand, a clear-cut suppressive effect of

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Analysis of GAL4-CREB fusions by cotransfection assay. A. Structure of GAL4-CREB fusions and reporter gene. B. Jurkat cells were cotransfected with the pG4-CAT reporter gene and a gene encoding GAL4\textsubscript{1-147}, GAL4-CREB\textsubscript{ΔLZ}, and GAL4-CREB\textsubscript{ΔLZM1}, then treated with extracellular Tat (10 ng/ml), p24 (10 ng/ml), or forskolin (10 μM). CAT promoter activity was measured as folds of activation with respect to Jurkat cells left untreated (medium alone). Data are reported as means ± SD of three to six independent transfection experiments performed in duplicate.
FIGURE 7. Western blot analysis of Ser^{333}CREB (A and C) and whole CREB protein (B and D), performed in freshly isolated PBMC pretreated with various pharmacologic inhibitors and then stimulated with Tat. A and B, PBMC were either left untreated (lane 1) or stimulated with Tat (10 ng/ml) for 30 min (lanes 2–8) in the absence (lane 2) or presence of 100 nM (lane 3), 500 nM (lane 4), or 1000 nM (lane 5) of wortmannin; or 50 nM (lane 6), 250 nM (lane 7), or 500 nM (lane 8) of staurosorpin. C and D, PBMC were either left untreated (lane 1) or stimulated with Tat (10 ng/ml) for 30 min in the absence (lane 2) or presence of 10 (lane 3) or 50 μM (lane 4) of PD 98059. A representative of five (A–B) or three (C–D) separate experiments is shown. The densitometric analysis is expressed in arbitrary units (a.u.).

The Tat-mediated CREB phosphorylation was noticed with staurosporin, a broad-spectrum protein kinase inhibitor and PD 98059, a specific MAPK inhibitor. Moreover, extracellular Tat stimulates the catalytic activity of MAPK showing a kinetics of activation (peak at 5–15 min) fully compatible with the Tat-mediated Ser^{333} CREB phosphorylation (peak at 30–60 min). Although these data suggest that extracellular HIV-1 Tat protein acts through an intracellular signal cascade after surface interactions with high affinity or integrin receptors (30–34), it is still possible that uptake of intracellular Tat may contribute to the induction of CREB phosphorylation. This issue clearly requires further investigation.

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References


