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p73 Interacts with Human Immunodeficiency Virus Type 1 Tat in Astrocytic Cells and Prevents Its Acetylation on Lysine 28

Shohreh Amini,[1] Giuseppe Mameli,[2] Luis Del Valle,[2] Anna Skowronska,[2] Krzysztof Reiss,[2] Benjamin B. Gelman,[3] Martyn K. White,[2] Kamel Khalili,[2] and Bassel E. Sawaya[2]*

Center for Neurovirology, Department of Biology,[1] and Center for Neurovirology, Department of Neuroscience, School of Medicine,[2] Temple University, 1900 North 12th Street, 015-96, Philadelphia, Pennsylvania 19122, and Texas Center for NeuroAIDS Research, Department of Pathology, University of Texas Medical Branch at Galveston, Rt. 0785, Galveston, Texas 77555-0785[3]

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Human immunodeficiency virus type 1 (HIV-1) Tat is a potent transcriptional activator of the HIV-1 promoter and also has the ability to modulate a number of cellular regulatory circuits including apoptosis. Tat exerts its effects through interaction with viral as well as cellular proteins. Here, we studied the influence of p73, a protein that is implicated in apoptosis and cell cycle control, on Tat functions in the central nervous system. Protein interaction studies using immunoprecipitation followed by Western blot and glutathione *S*-transferase pull-down assays demonstrated the association of Tat with p73. Tat bound to the N-terminal region of p73 spanning amino acids 1 to 120, and this interaction required the cysteine-rich domain (amino acids 30 to 40) of Tat. Association of p73 with Tat prevented the acetylation of Tat on lysine 28 by PCAF. Functional studies including RNA interference showed that p73 inhibited Tat stimulation of the HIV-1 promoter. Furthermore, p73 prevented the interaction of Tat with cyclin T1 in vitro but not in vivo. These findings suggest possible new therapeutic approaches, using p73, for Tat-mediated AIDS pathogenesis.

Human immunodeficiency virus type 1 (HIV-1) affects the central nervous system (CNS) of a majority of AIDS patients and often leads to neurological symptoms such as memory loss and impaired cognitive and motor functions (45). More than half of pediatric AIDS patients and almost 20 to 25% of HIV-infected adults eventually develop dementia (43). HIV-associated neuropathology includes reactive astrocytosis, cerebellar atrophy in the early stage of infection, demyelination, formation of multinucleated giant cells, neuronal death, and breakdown of the blood-brain barrier at the late stages of the disease (13). The primary cells for HIV-1 infection in the brain are microglia/macrophages and astrocytes. The neuronal cells which may not support productive viral infection exhibit severe pathology, presumably due to an indirect pathway that involves secretion of viral and cellular factors from the infected cells, all contributing to the neuropathogenesis of AIDS (39). In this regard, HIV-1 Tat has been proposed as one of the key neuropathogenic factors which can induce oxidative stress in the infected cells (16) and stimulate viral gene transcription from the proviral long terminal repeat (LTR) by interacting with several cellular factors (46). One such factor is the Tat-associated kinase CDK9/P-TEFb, which is recruited by Tat on the *trans* activation response (TAR) element located at the 5′ termini of all viral transcripts. This complex stimulates transcriptional elongation by phosphorylating the RNA polymerase II C-terminal domain (52). Tat is a small protein of 101 amino acids (aa), but because of a single nucleotide change in LAI, HXB2, and/or pNL4-3 HIV-1 strain isolates at putative residue 87, most of the structural studies were made with a Tat protein composed of 86 residues (18). The linear amino acid sequence of Tat can be divided into several domains including the acidic domain and the cysteine-rich region (aa 21 to 37), which is important for dimerization of Tat and transactivation of HIV-1 LTR. This region might also be implicated in Tat-mediated apoptosis and seems to be critical for Tat-induced neurotoxicity (35). Tat also contains a basic domain (aa 48 to 57) that is critical for nuclear localization of Tat and its binding to the TAR domain (55). In addition, the C-terminal portion of Tat contains the tripeptide sequence RGD (Arg-Gly-Asp) that binds to cell surface integrin receptors and mediates Tat uptake by uninfected cells (10). A number of cellular proteins have been reported to interact directly with Tat, including p-TEFb (46), p300 (17), p300/CBP-associated factor (PCAF) (49), and the tumor suppressor p53 (29).

Given the importance of p53 in regulating cell growth and apoptosis in response to various cellular stress signals (26), a significant effort has been put forth to identify p53 homologues. Several p53-like proteins have been identified, including the p73 gene product (23). Due to its considerable homology to p53, initial speculation was that p73 might function similarly to p53, causing apoptosis and growth arrest (19). In addition, the importance of p73 as a tumor suppressor was suggested by its location on chromosome 1p36, a region frequently deleted in neuroblastoma and other tumors (23). It should be noted that p73 is expressed monoallelically in neuroblastomas, so its tumor suppressor function remains unclear, since only the wild-type form has been identified in all tumors or tumor cell lines tested. Alternative splicing of the human p73 gene generates at least six variants, α, β, δ, γ, ε, and ζ (27). p73β is composed of 499 amino acids, which makes it 137 amino acids shorter than p73α (636 aa) (23). Genetic studies of

* Corresponding author. Mailing address: Center for Neurovirology and Cancer Biology, Temple University, 1900 North 12th Street (015-96), Philadelphia, PA 19122. Phone: (215) 204-0607. Fax: (215) 204-0679. E-mail: sawaya@temple.edu.

the p73 protein revealed three functional domains, including a transactivation domain (residues 1 to 54), a DNA binding domain (residues 131 to 310), and an oligomerization domain (residues 345 to 380), which are common to the α and β variants of p73. Overexpression of p73 induces the transcription of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ (21) and several other genes involved in apoptosis and cell cycle control. Furthermore, p73 can be phosphorylated (11); it interacts with Mdm2 (27) and can be degraded by SUMO-1 (30).

Unlike p53, p73 is not induced by DNA damage signals, suggesting a distinct pathway/role for this protein in response to stress. In light of previous observations on the role of Tat in CNS cell dysfunction, and its interaction with p53, we investigated the possible interaction between p73 and Tat in brain cells.

MATERIALS AND METHODS

Plasmids. HIV-1 LTR-luciferase (Luc) reporter plasmid cytomegalovirus (CMV)-Tat (86 aa) and its deletion mutant, CMV-Tat(Δ2–36); glutathione *S*-transferase (GST)-Tat (86 aa) and its various deletion mutants; and enhanced cyan fluorescent protein (CFP) (ECFP)-Tat (86 aa) plasmids were described previously (36). Rous sarcoma virus–HIV-1 Tat_{K28A}, Tat_{K50A}, and $Tat_{K28A/K50A}$ were kindly provided by R. Harrod (Southern Methodist University, Dallas, TX) (49). Each of these was recloned into pcDNA3 using the BamHI and EcoRI sites. The Bax-Luc reporter plasmid was a gift from J. Manfredi (Mount Sinai School of Medicine, NY). The pDsRed1-mito plasmid was purchased from Clontech (Paolo Alto, CA). The pcDNA3-p300 plasmid was a gift from A. Giordano (Temple University, Philadelphia, PA). GST-p300 histone acetyltransferase (HAT) was kindly provided by F. Kashanchi (George Washington University, Washington, D.C.). PCAF-Flag, $pcDNA_3$-p73α, and $pcDNA_3$-p73β expression vectors were a gift from Y. Nakatani and W. G. Kaelin, Jr. (Dana Farber Cancer Institute, MA). To create GST-PCAF HAT, we generated a 1,443-bp fragment by PCR from PCAF-Flag which corresponds to the region encompassing aa 351 to 832 and then inserted it into the BamHI-EcoRI sites of pGEX-5. The hemagglutinin (HA)-p73α deletion mutants (566, 526, 425, and 246 aa) were excised from the 636-aa full-length p73α using the following restriction enzymes: NotI-XbaI, EcoNI-XbaI, HincII-XbaI, and EcoRI-XbaI, respectively. The resulting products were then blunt ended with the Klenow fragment and self-ligated. The full-length GST-p73 and deletion mutants were amplified by PCR and cloned into the BamHI-XhoI site of pGEX-5X1. The sequences of the oligonucleotides (numbers in parentheses refer to amino acid positions in p73 as shown in Fig. 3) used in PCR are as follows: (i) 5′-GCCGGCGGGATCCAGATGGCCCAGTC CACC-3′ (1/120, 1/321, 1/494, and 1/636), (ii) 5′-CCCCTCCGGATCCACTAC CCCGGACCCCAC-3′ (120/321), (iii) 5′-GAGAGCTGGATCCAGAACGGG GCCGCCAGC-3′ (321/636), (iv) 5′-GAAAGTGACCTCGAGGTGGTGGGG GTCCGG-3′ (120/1), (v) 5′-GCACGCTTGCTCGAGGCCCCGTTCTTGGC G-3′ (321/1 and 321/120), (vi) 5′-ATGGTTCTCGAGCATCCCGGGGCCCAC GGG-3′ (494/1), and (vii) 5′-GCGAGGCTCGAGGTGGATCTCGGCCTCCG T-3′ (636/1).

Cell culture, transfection, and luciferase assays. The human astrocytic glial cell line U-87MG was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc., CA). The HL3T1 HeLa-derived cell line contains stably integrated, silent copies of the HIV-1 LTR promoter linked to the chloramphenicol acetyltransferase (CAT) gene. CAT expression is induced upon introduction of active Tat (50). Cells (5×10^5) cultured on 60-mm plates and grown overnight were transfected using precipitation by the calcium phosphate method. The cells were transfected with 0.1 μg of the reporter plasmids (LTR-Luc or p73-Luc) alone or in combination with 0.5 μg of various expression vectors. To control the concentration of the promoter DNA sequence in transfection, $pcDNA_3$ empty plasmid was added to the transfection mixture. Each transfection was repeated a minimum of three separate times with at least three different plasmid preparations. Cell extracts were prepared 48 h after transfection, and luciferase assays were performed according to the manufacturer's instructions (Promega, Madison, WI).

Western blot analysis and antibodies. Ten micrograms of a plasmid encoding pECFP-Tat or $pcDNA_3$-Tat (full length or mutant) or 10 μg of a plasmid encoding $pcDNA_3$-p73 or both were transiently introduced into U-87MG cells. Forty-eight hours posttransfection, Western blotting was performed on total cell lysates (50 μg/sample) as described previously (37). Antibodies against Flag, cyclin T1, p73 (C-20, which recognizes p73α and p73β), and Grb2 were used to detect the level of these proteins (Santa Cruz, CA).

GST pull-down assays. GST, GST-Tat, and GST-p73, and the deletion mutant fusion proteins were prokaryotically expressed and purified as described previously (2). Radiolabeled Tat, p73α, and p73β proteins were synthesized with a TNT-coupled wheat germ extract system according to the manufacturer's recommendations (Promega, Madison, WI). For in vitro binding assays, 4 μl of [^{35}S]methionine-labeled and translated proteins was incubated with 5 μg of GST or GST fusion proteins, as indicated in the figures, coupled to glutathione-Sepharose beads in 300 μl of lysis buffer 150. The bound proteins were eluted with Laemmli sample buffer, heated to 95°C for 5 min, and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

In vitro acetylation assay. HAT assay was performed as described previously (32). Briefly, 30 μl of the total reaction mixture was incubated at 30°C for 1 h in a buffer containing 1 μg of purified GST, GST-p300 HAT, or GST-PCAF HAT; 0.5 μg of substrate of purified Tat and/or p73 proteins; 50 mM HEPES, pH 8.0; 10% glycerol; 10 mM sodium butyric acid; 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; and 1 μl of [^{14}C]acetyl coenzyme A (55mCi/mmol; Amersham, NJ). Proteins were resolved by 15% SDS-PAGE. After incubation in a solution containing 10% glacial acetic acid and 40% methanol for 1 h, the gel was then dried and exposed to X-ray film for several days.

Immunocytochemistry. Cells were plated on poly-L-lysine-coated glass chamber slides and allowed to attach overnight. U-87MG cells were transfected with 1.0 μg of pDsRed1-mito plasmid targeted to the mitochondrial matrix (Clontech, Paolo Alto, CA) in the presence or absence of 2.5 μg of ECFP-Tat. Cells were then fixed for 3 min in ice-cold acetone, followed by washing with phosphate-buffered saline (PBS). After blocking with 2% normal rabbit serum for 2 h, slides were incubated in primary antibody (p73) overnight at room temperature. Cells were then washed in PBS, incubated in anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody for 2 h at room temperature in the dark, rinsed with PBS, and mounted in an aqueous mounting medium (Vector Laboratories, Burlingame, CA).

RNA interference. SmartPool small interfering RNA (siRNA) against p73 (siRNA-p73) (Dharmacon, Lafayette, CO) was transfected at a concentration of 400 nM into approximately 1×10^6 U-87MG cells in serum-free Optimem (Invitrogen) alone or in the presence of 1 μg of plasmids that express Tat, p73α, or p73β by using Lipofectamine transfection reagents (Roche Molecular Biochemicals).

ChIP assay. HL3T1 cells were grown overnight in 100-mm dishes to 60 to 70% confluence; cells were then transfected with 1 μg of CMV-Tat, CMV-p73α, and/or CMV-p73β expression plasmids using various combinations by using a FuGENE 6 transfection reagent (Roche Applied Sciences). Plates were returned to the incubator for 40 to 48 h. At this time, cells were cross-linked with formaldehyde and harvested, and chromatin immunoprecipitation (IP) (ChIP) was performed. For these studies, only 5×10^6 cells were used per immunoprecipitation reaction because the plasmid is present at a high copy number. The remainder of the procedure followed standard protocols for ChIP analysis, as has been described previously (48). The resulting DNA was analyzed by PCRs using the following HIV LTR primers: 5′-AACTGGTACCATCGAGCTTGCT-3′ (forward) (−120/+66) and 5′-TTGAG GATCCAGCAGTGGGTTC-3′ (reverse) (+66/−120). Antibody used in the ChIP procedure (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was p73 as well as rabbit anti-mouse immunoglobulin G.

RESULTS

Association of p73 with Tat in vitro and in cells. The existence of physical and functional interplay between Tat and p53 (28, 29) prompted us to determine whether Tat and p73 can physically interact. U-87MG cells were transfected with plasmids encoding Tat (Fig. 1B), CFP-Tat (Fig. 1C), HA-p73α, and/or HA-p73β. Protein extracts derived from these cells were subjected to Western analysis to verify the expression of HA-p73 and CFP-Tat proteins, especially since tagged p73 (HA-p73), Tat, or CFP-Tat proteins are not endogenously expressed. To that end, we performed 8% or 12% SDS-PAGE to verify the expression of HA-p73 or CFP-Tat, respectively. As shown in Fig. 1A, anti-HA or anti-Tat antibodies confirmed the ectopic expression of HA-p73 (lanes 1 and 2) or CFP-Tat (lanes 3 and 4) proteins, respectively. Next, we performed a

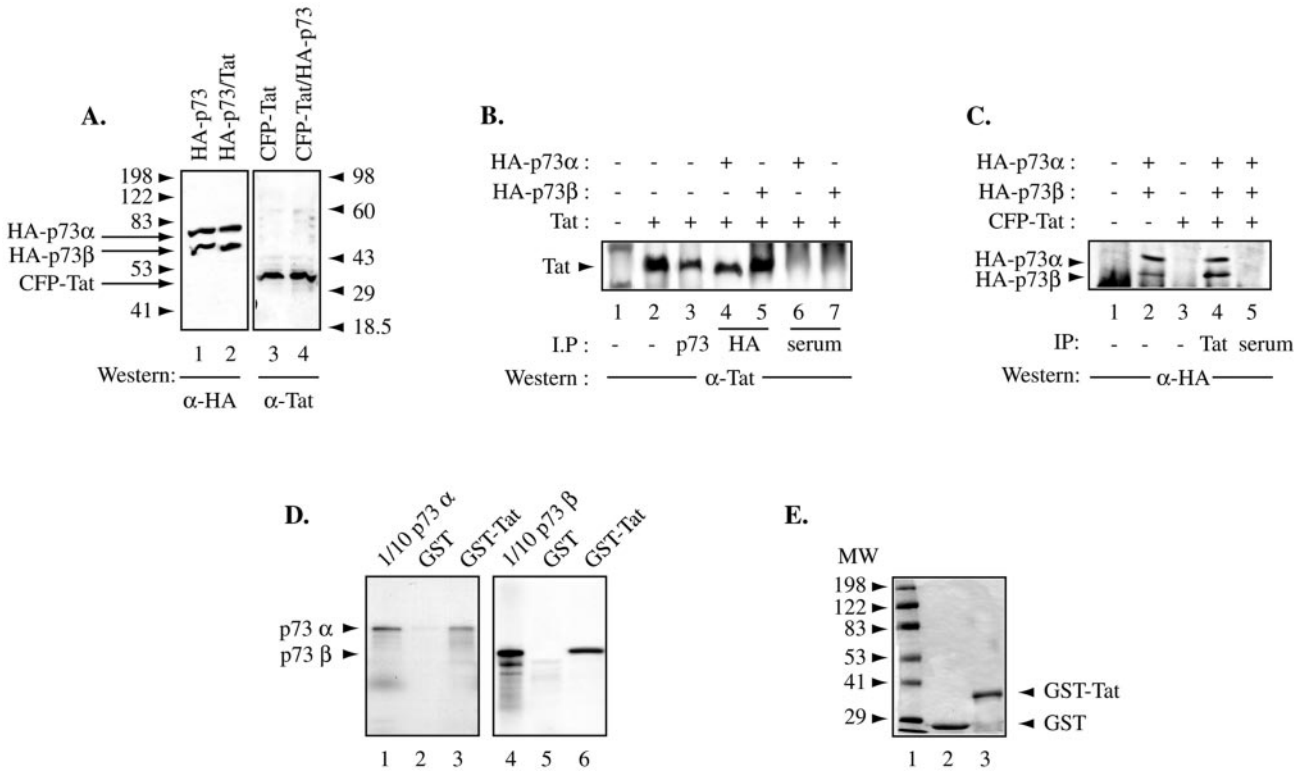

FIG. 1. Association of Tat and p73 proteins in vivo and in vitro. (A, B, and C) Cell lysates were prepared from U-87MG cells transfected with plasmids expressing different cDNA constructs as indicated above A, B, and C. Fifty micrograms was used for Western analysis to verify the expression of HA-p73 (A, lanes 1 and 2) or CFP-Tat (A, lanes 3 and 4). Approximately 300 μg of cell extract was utilized in immunoprecipitations (IP) followed by Western blot utilizing anti-Tat (α-Tat) or rabbit serum and anti-HA (α-HA) antibodies, respectively (B, lanes 4 and 5), or anti-p73, anti-HA, or rabbit serum for IP followed by anti-Tat (C, lanes 3, 4, 5, 6, and 7, respectively). In parallel, 50 μg of extracts was utilized by direct Western blot assay (B, lanes 1 to 3 and lanes 1 and 2, respectively). The arrows depict the positions of the 73- and 66-kDa p73α and p73β (B) and the 13-kDa (C) Tat. The Western or IP/Western assays were carried out according to the procedure described previously (2). (D) In vitro-translated [^{35}S]methionine-labeled wild-type p73α (lanes 1 to 3) and p73β (lanes 4 to 6) were incubated with either GST (lanes 2 and 5) or GST-Tat (lanes 3 and 6) as indicated above the lanes. The positions of the 73- and 66-kDa p73 bands bound to the GST fusion proteins are shown. (E) Stained SDS gel showing the quality and the size of GST or GST-Tat fusion proteins. MW, molecular weight (in thousands).

coimmunoprecipitation procedure with a polyclonal anti-HA or anti-Tat antibody followed by Western blot analysis (Fig. 1B and C, respectively). As shown in Fig. 1, proteins isolated from nontransfected (lanes 1) or transfected cells (lanes 2) were used as negative and positive controls, respectively (Fig. 1B and C, lanes 1 and 2). Extracts were coprecipitated with polyclonal anti-p73 antibody (to detect the endogenous p73) (Fig. 1B, lane 3), with anti-HA antibody (to detect the overexpressed p73) (Fig. 1B, lanes 4 and 5), or with rabbit serum (Fig. 1B, lanes 6 and 7). Western blot analysis of the precipitate confirmed the association of Tat with the endogenous p73 (lane 3) or between Tat and the overexpressed p73 (lanes 4 and 5). The association between Tat and p73 is specific, as shown by the use of preimmune rabbit serum (Fig. 1B, lanes 6 and 7). Reciprocally, extracts prepared from Tat-transfected cells did not react or cross-link with anti-HA antibody (Fig. 1C, lane 3). The p73 proteins were specifically coprecipitated by the polyclonal anti-Tat antibody (Fig. 1C, lane 4). The specificity of the interaction between Tat and p73 was shown by the use of preimmune rabbit serum (Fig. 1C, lane 5).

The ability of Tat to interact with p73 was also tested by GST pull-down assay using in vitro-translated (IVT) [^{35}S]methionine-labeled p73α and/or p73β. Labeled proteins were incubated with GST or the GST-Tat fusion protein coupled to glutathione-Sepharose beads. As shown in Fig. 1D, p73α and p73β were retained by the GST-Tat fusion protein but not by GST alone (Fig. 1D, compare lanes 2 to 3 and 5 to 6). Figure 1E illustrates the size of GST (lane 2) or GST-Tat (lane 3), respectively.

Mapping of p73-interacting domains within Tat. In the next series of experiments, we aimed to determine the domain of Tat involved in interaction with p73. The technique of GST pull-down assay was used. A series of N- and C-terminal deletion mutants of HIV-Tat (Fig. 2A) were fused to GST and incubated with purified recombinant p73 as described in Materials and Methods. As shown in Fig. 2A, deletion of the N- or C-terminal regions of Tat up to residues 30 and 40, respectively, did not affect its ability to bind to p73α or p73β. Instead, deletion of residues 30 to 40 abrogated the interaction of p73 with Tat (Fig. 2A). These observations suggest that residues 30 to 40 of Tat are crucial for its association with p73α and/or p73β. Figure 2B and C illustrate a representative result from a GST pull-down assay obtained with p73α and p73β. Figure 2D illustrates the size of GST (lane 2) or GST-Tat deletion mutants (lanes 3 to 10), respectively.

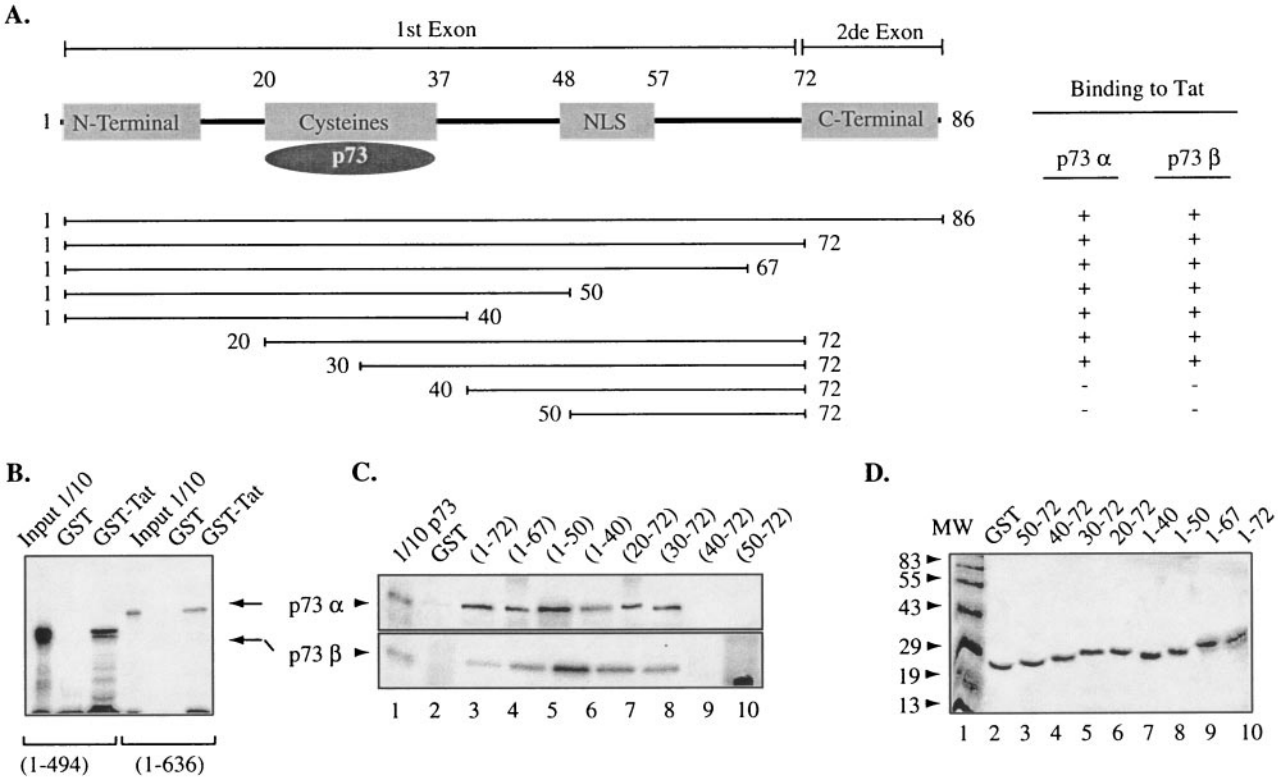

FIG. 2. Identification of the Tat domain that binds to p73α or p73β. (A) Schematic representation of wild-type Tat and its deletion mutants. Binding abilities of p73α or p73β to various Tat mutants are shown on the right. B and C illustrate a representative result from GST pull-down assay obtained with p73α and p73β. Wild-type Tat or the indicated mutants fused to GST and immobilized on glutathione-Sepharose were incubated with in vitro-synthesized [^{35}S]methionine-labeled p73α or p73β proteins. After incubation for 2 h at 4°C, the bound proteins were eluted and analyzed by SDS-PAGE. (D) Stained SDS gel showing the quality and the size of GST or GST-Tat deletion mutant fusion proteins. NLS points to the nuclear localization signal domain. MW, molecular weight (in thousands).

Mapping of Tat-interacting domains within p73. Reciprocally, to map the Tat binding site within the p73 protein, we carried out a similar set of GST pull-down assays using either in vitro-transcribed/translated p73 deletion mutant proteins or GST-p73 fusion proteins. We have generated a series of p73 deletion mutants in which indicated domains were deleted (Fig. 3A, B, and C). The p73 deletion mutant proteins were labeled with [^{35}S]methionine and incubated with GST or GST-Tat fusion protein. As shown in Fig. 3B, the region encompassing amino acids 1 to 246 within p73 is the region involved in binding with Tat. This region has been shown to contain the transactivation domain of p73 (23). To further confirm the importance of the N-terminal domain of p73 in the interaction with Tat, GST pull-down was carried out using p73 deletion mutants in which N- or C-terminal domains were deleted. Tat was not retained by GST or by GST-p73ΔN fusion protein (Fig. 3C). Thus, Tat appears to recognize the N-terminal domain of p73, which shares 29% identity with the p53 N-terminal domain (residues 1 to 58). Our data corroborate previous findings pointing to the interaction between the N-terminal domain of p73 and other proteins (54). Similar results were obtained with p73βΔ-N (data not shown). Figure 3D and E illustrate a representative result from GST pull-down assay obtained with p73α. Fig. 3F illustrates the size of GST (lane 2) or full-length GST-p73 and GST-p73 deletion mutants (lanes 3 to 8), respectively.

Effect of Tat-p73 interaction on HIV-1 Tat's functions. To determine the functional relevance of p73-Tat association, we studied the effect of p73 on Tat's various functions. First, we evaluated the effect of p73 on HIV gene expression in the presence and absence of Tat. U-87MG cells were transfected with the reporter plasmid (LTR-Luc) alone or in combination with either p73α or p73β. As shown in Fig. 4A, transfection of either p73α or p73β leads to 10.40- and 5.99-fold activation of the HIV-1 promoter, respectively (compare lane 1 to lanes 2 and 3). Transfection of CMV-Tat leads to 21.3-fold activation of HIV-1 LTR (lane 4). When the transfection was performed using different combinations of Tat-p73α or Tat-p73β, activation of the LTR was lower than that observed with individual plasmids (6.44- and 3.49-fold) (Fig. 4A, compare lane 4 to lanes 5 and 6).

The ability of p73 to interact with Tat and to affect Tat activation of HIV LTR prompted us to examine whether p73 has the potential to bind HIV LTR sequences. To this end, ChIP assays were performed. The HL3T1 cell line, which contains integrated HIV LTR CAT, was transfected with Tat, p73α, and p73β expression plasmids using various combinations. Forty-eight hours after transfection, the cells were har-

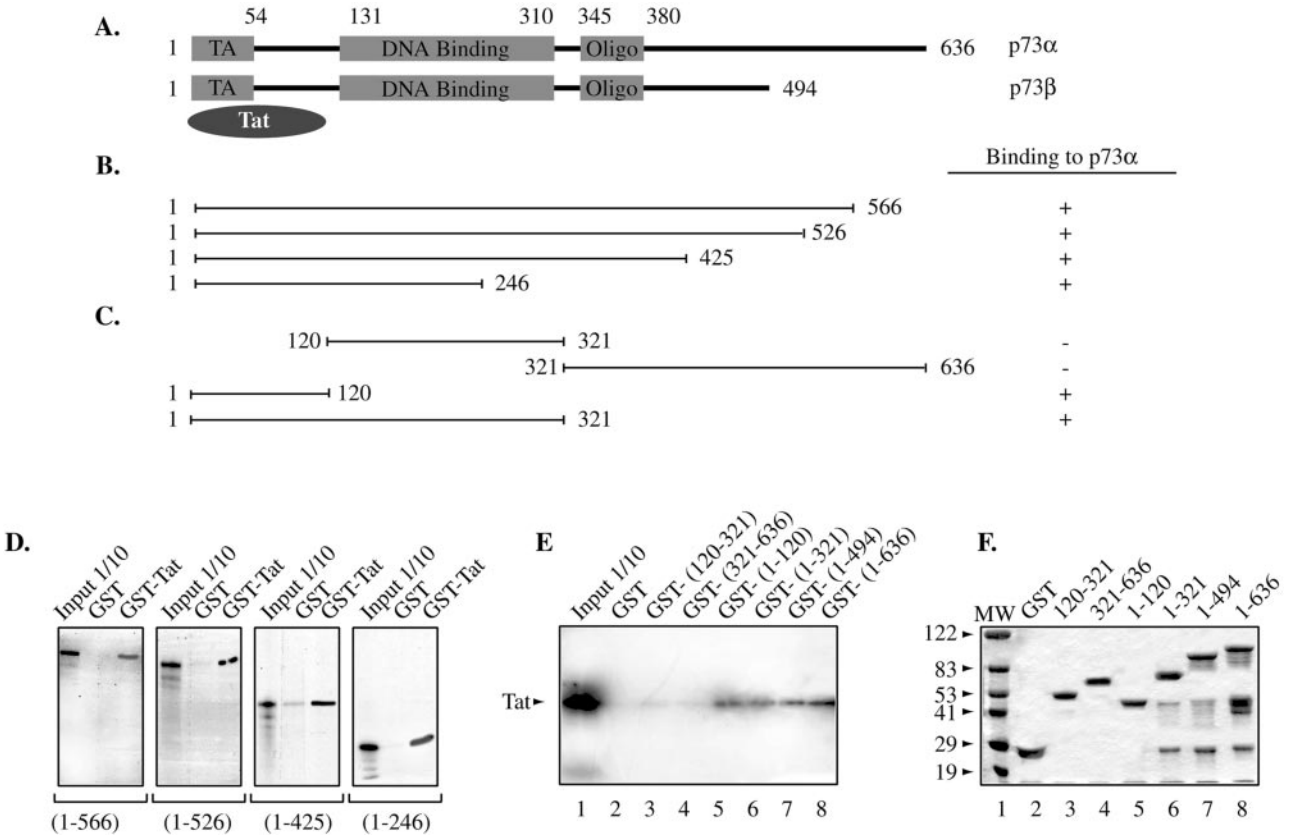

FIG. 3. Tat interacts with the N-terminal domain of p73. (A) Schematic representation of p73α and p73β with their important domains. (B and C) Deletion mutants of p73α used in this study as in vitro-translated proteins (B) or fused to GST (C). Binding abilities of Tat to various p73α mutants are shown on the right (B). D and E illustrate representative results from GST pull-down assay obtained with IVT [^{35}S]methionine-labeled p73α (D) or with GST-p73α (E). (F) Stained SDS gel showing the quality and the size of GST or GST-p73 (full length and deletion mutants) fusion proteins. MW, molecular weight (in thousands).

vested and subjected to ChIP using anti-p73 antibody as described in Materials and Methods. Rabbit anti-mouse serum was used as a negative control. As shown in Fig. 4B, endogenous as well as transfected p73 had the ability to bind HIV LTR sequences (lane 4). No interaction was observed when the nonspecific control serum was used (lane 3). p73-DNA interaction was not affected by the presence of Tat (Fig. 4B, lanes 4). These data demonstrated that beyond its interaction with Tat protein, p73 also interacts with HIV LTR sequences. Further studies are needed to determine the p73-responsive element within HIV LTR.

The interplay between Tat and p73 was further studied in the absence of p73 using siRNA directed against p73. Cells were transfected with 400 nM siRNA directed against p73 alone or in the presence of 1 μg of pcDNA$_3$, pcDNA$_3$-p73α, and/or pcDNA$_3$-p73β expression plasmids. Twenty-four hours after transfection, the cells were collected and processed for Western analysis. As shown in Fig. 4C, p73 (α or β) was completely silenced in extracts prepared from siRNA-transfected cells (compare lanes 2 to 5 and 3 to 6). Note that pcDNA$_3$ was used as a negative control (lanes 1 and 4).

HL3T1 cells were then transfected with CMV-Tat in the presence and absence of siRNA-p73. Twenty-four hours after transfection, the cells were collected and processed for CAT assays. Interestingly, activation of HIV-1 LTR by Tat was greater in the presence of siRNA-p73 (compare lane 2 to lane 4). Our results led us to conclude that although Tat activates HIV LTR, this activation can be increased in the absence of endogenous p73.

The p73 protein was previously shown to activate expression of the Bax promoter (44). Therefore, to determine whether the effects of Tat and p73 were LTR specific, we expanded our analysis to the Bax promoter. As shown in Fig. 4E, transfection of Bax reporter plasmid alone or in combination with Tat (lane 2), p73α (lane 3), or p73β (lane 4) resulted in an increase in Bax gene expression. The transfection assays were then performed using different combinations of plasmids: Tat-p73α or Tat-p73β. A decrease in the activation of the Bax promoter was observed when Tat was coexpressed with either p73α (1.09 versus 3.63) (lane 5) or p73β (0.75 versus 2.39) (Fig. 4E, lane 6). Note that the cells were transfected in triplicate in which one dish was used to verify the efficiency of transfection, which was examined by Western blot analysis as shown in Fig. 4A and E. Grb2 was used to verify the equal loading of cellular proteins.

Results from cotransfection studies indicate that Tat impaired p73 activation of HIV-1 LTR and Bax promoters and vice versa. Similar results were obtained with the p21^{WAF1}

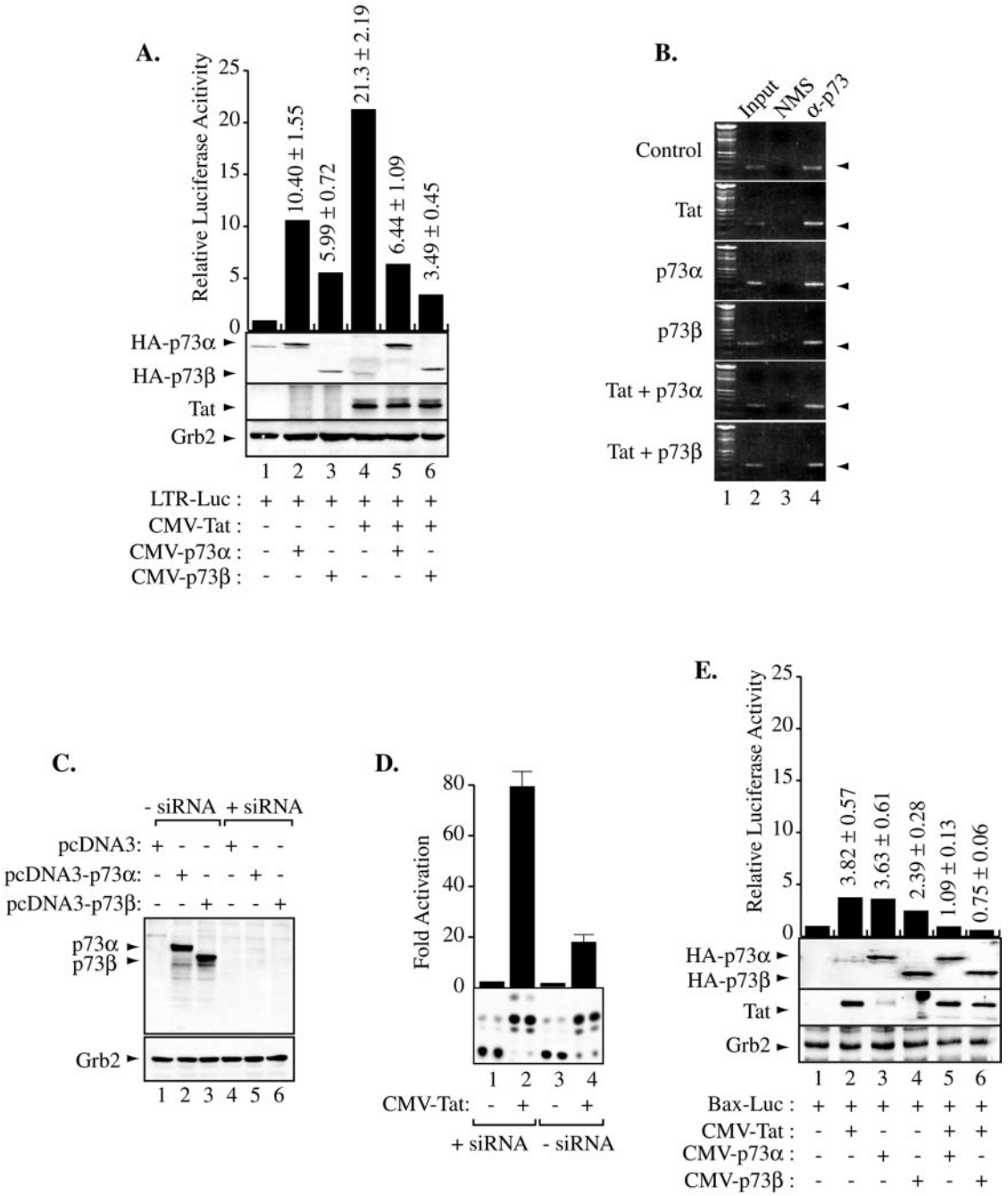

FIG. 4. Effect of p73-Tat association on Tat function. (A and E) U-87MG cells were transfected with 0.1 μg of the reporter plasmids (LTR or Bax-Luc) alone or in combination with 0.5 μg of Tat, p73α, or p73β expression plasmids, separately or combined, as indicated. Total amounts of transfected DNA were maintained constant by the addition of empty control vector. Cell extracts were prepared 48 h after transfection, and luciferase activity was determined. The data represent the mean value of three separate transfection experiments. Protein expression as well as equal protein loading were examined by Western blot assays using anti-p73, anti-Tat, and anti-Grb2 antibodies. (B) HL3T1 cells were transfected with 1.0 μg of Tat, p73α, or p73β expression plasmids, separately or combined, as indicated. Interaction of p73 with HIV LTR DNA was demonstrated by ChIP assays. The primers used in these experiments are described in Materials and Methods. Anti-p73 (lane 4), preimmune serum (lane 3), and no antibody (lane 2) were used in these experiments. NMS, normal mouse serum. (C) HL3T1 cells were transfected with siRNA-p73 (lanes 4 to 6) and/or CMV-p73 (lanes 2, 3, 5, and 6). Approximately 50 μg of extracts was utilized in the Western blot assay utilizing anti-p73 or anti-Grb2 antibody, respectively. The arrows depict the positions of the different proteins. Cells transfected only with pcDNA3 were utilized as negative controls (lanes 1 and 4). (D) HL3T1 cells were transfected with 0.5 μg of Tat expression plasmid in the presence and absence of siRNA p73. Total amounts of transfected DNA were maintained constant by the addition of empty control vector. Cell extracts were prepared 48 h after transfection, and CAT activity was determined. The data represent the mean values of three separate transfection experiments.

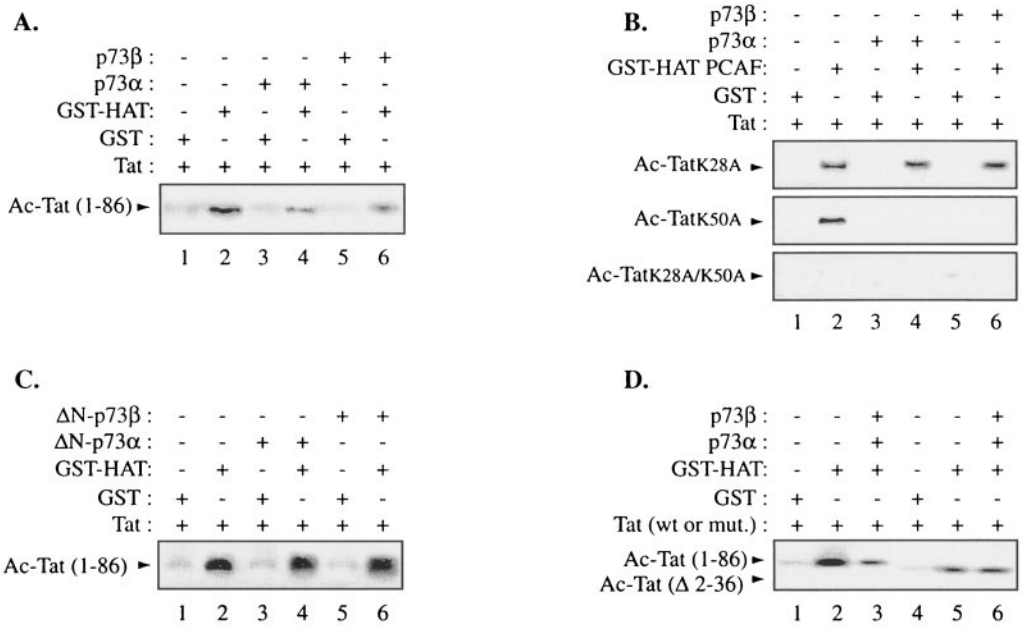

FIG. 5. Effect of p73 on Tat acetylation. (A to D) Full-length (wild-type [wt]) or mutant (mut.) Tat and/or p73 (full-length or deletion mutant) proteins were incubated with GST (A to C, lanes 1, 3, and 5, and D, lanes 1 and 4) or GST-HAT (from p300) (A and C, lanes 2, 4, and 6, and B, lanes 2, 3, 5, and 6) or GST-HAT (from PCAF) (B, lanes 2, 4, and 6) and [^{14}C]acetyl coenzyme A. Acetylated (Ac) products were resolved by 15% SDS-PAGE, dried, and exposed to X-ray film.

promoter (data not shown), indicating reciprocal attenuation of Tat and p73 transcriptional activities.

Effect of Tat-p73 interaction on Tat acetylation. Previously, it has been shown that PCAF binds to the cysteine-rich region of Tat and promotes its acetylation on lysine 28 (18). Acetylated Tat becomes functional and activates HIV-1 LTR expression (22, 49). Therefore, the association between Tat and p73 might prevent the acetylation of Tat by PCAF. To examine this hypothesis, we incubated Tat protein with the HAT domain of p300 fused to GST in the presence and absence of in vitro-translated p73. As shown in Fig. 5A, GST-HAT but not GST was able to acetylate Tat (compare lanes 1 and 2). Surprisingly, in the presence of either IVT p73α or IVT p73β, acetylation of Tat by GST-HAT was much less than that in their absence (compare lane 2 to lanes 4 and 6).

Tat can be acetylated at lysines 28 and 50; therefore, the weak acetylation observed in the presence of p73 might be due to acetylated lysine 50 (22). To verify this hypothesis, full-length Tat in which lysine 28, lysine 50, or lysines 28 and 50 were mutated to alanine was used and incubated with GST or GST-HAT from PCAF. As shown in Fig. 5B, in the absence of lysine 28, Tat_{K28A} remains acetylated in the presence and absence of IVT p73α or IVT p73β (lanes 2, 4, and 6). In the absence of lysine 50, Tat_{K50A} remains acetylated in the absence of p73 but not in its presence (compare lane 2 to lanes 4 and 6). When $Tat_{K28A/K50A}$ was used, no acetylation was observed in the presence or absence of p73. These data led us to conclude that association of p73 with Tat prevents acetylation of lysine 28 within Tat.

Next, we examined the acetylation status of Tat in the presence of p73 mutants that lack the N-terminal domain (ΔN-p73α or ΔN-p73β). As shown in Fig. 5C, IVT ΔN-p73α or IVT-ΔN-p73β failed to affect the acetylation of Tat (compare lanes 2, 4, and 6). These data correlate with our observations in which we demonstrated that physical association between Tat and p73 requires the N-terminal domain of p73.

Reciprocally, we examined the ability of p73 to weaken the acetylation of Tat in the absence of a p73-binding domain within Tat. To that end, a Tat mutant [Tat(Δ2–36)] was incubated with GST-HAT in the presence and absence of IVT p73α or IVT p73β. As shown in Fig. 5D, the acetylation level of wild-type Tat (compare lanes 2 and 3) but not that of Tat(Δ2–36) decreases in the presence of p73α and p73β (compare lanes 5 and 6). It should be noted that Tat_{K28A} remained associated with p73; therefore, the use of Tat(Δ2–36) was indispensable.

Functional interplay between p73 and Tat mutants. The ability of p73 to prevent Tat acetylation on lysine 28 prompted us to study the functional interplay between p73 and Tat mutants in which lysine 28 and lysine 50 were mutated to alanine. U-87MG cells were transfected with LTR-Luc alone or in the presence of Tat (full length or mutants), p73α, and/or p73β expression plasmids using various combinations. Tat mutants contained a single or double point mutation at lysine 28, lysine 50, or lysines 28 and 50 (K28A, K50A, and K28A/K50A). As shown in Fig. 6, full-length and mutant Tats, bearing single but not double point mutations, have the ability to activate HIV-1 LTR (lanes 2 to 5). The addition of p73α or p73β prevents full-length Tat and Tat mutant K50A from activating HIV LTR (Fig. 6, compare lanes 7, 9, 12, and 14 to lanes 2 and 4). Neither p73α nor p73β had any effect on Tat mutant K50A (Fig. 6 compare lanes 8 and 13 to lane 3). Expression of transfected plasmids was tested by Western blot assay, as shown in Fig. 6.

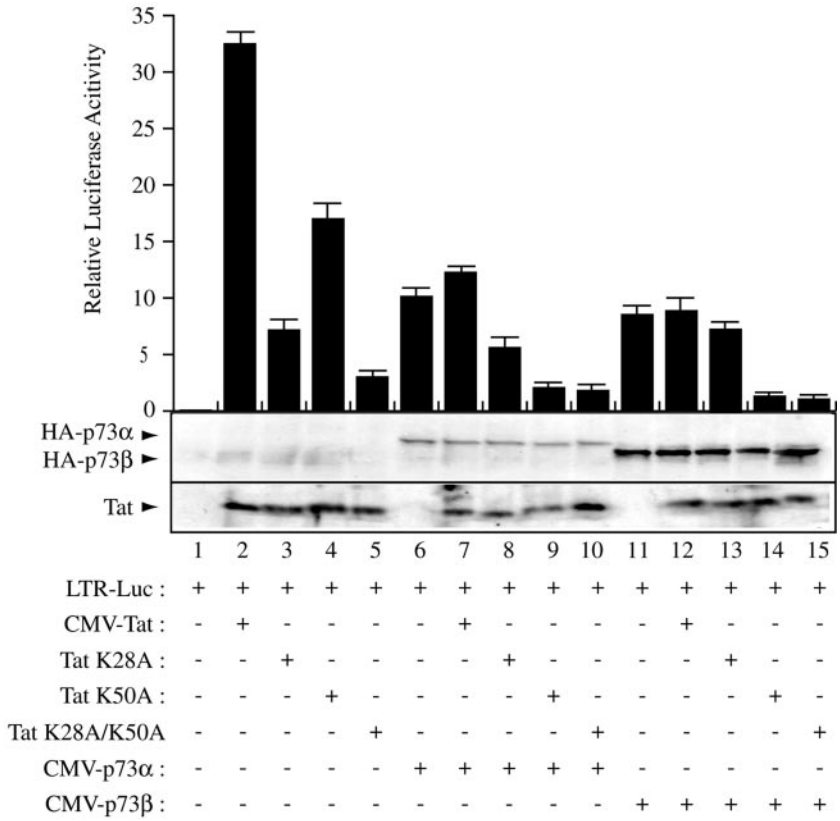

FIG. 6. Functional interplay between p73 and Tat mutants. U-87MG cells were transfected with 0.1 μg of the reporter LTR-Luc plasmid alone or in combination with 0.5 μg of Tat (full length or mutants), p73α, or p73β expression plasmids, separately or combined, as indicated. Total amounts of transfected DNA were maintained constant by the addition of empty control vector. The data represent the mean values of three separate transfection experiments. Cell extracts were prepared 48 h after transfection, and luciferase activity was determined. Protein expression as well as equal protein loading were examined by Western blot assays using anti-p73 and anti-Tat antibodies.

p73 weakens the interaction of Tat with PCAF. Several proteins, including p300, PCAF, and hGCN5, have been shown to acetylate Tat at lysine 28 and lysine 50 (6, 22, 24, 31). PCAF was shown to acetylate Tat at both lysine 28 and lysine 50 (24, 31), while p300 and hGCN5 were shown to acetylate Tat only at lysine 50 (6). Acetylation of Tat at lysine 28 enhances Tat binding to p-TEFb, while acetylation at lysine 50 promotes the dissociation of Tat from TAR RNA (49). These observations led us to investigate a potential competition between p73 and PCAF or between p73 and cyclin T1 to interact with Tat. To that end, U-87MG cells were transfected with plasmids encoding Tat, p73α/p73β, and/or PCAF-Flag. Protein from transfected cells was subjected to immunoprecipitation with a polyclonal anti-Flag or anti-p73 antibody followed by Western blot analysis (Fig. 7A). Proteins isolated from nontransfected cells (lanes 1) or Tat-transfected cells (lanes 2) were used as negative and positive controls, respectively. The level of detected Tat increased in extracts prepared from Tat/PCAF-transfected cells (lane 3). Cell extracts were coprecipitated with polyclonal anti-Flag antibody (lanes 4 and 6) or anti-p73 antibody (lane 5), with rabbit serum (lane 7), or with Sepharose beads (lane 8). Western blot analysis of the precipitate confirmed the existence of a direct association between Tat and PCAF in the absence of p73 (lane 4) and a very weak one in its presence (lane 6).

Given that p300 acetylates lysine 50 of Tat through their physical interaction, we examined the ability of Tat to interact with p300 in the presence of p73. U-87MG cells were transfected with plasmids encoding Tat, p73α/p73β, and/or p300. Proteins derived from the transfected cells were subjected to immunoprecipitation with a polyclonal anti-p300 or anti-p73 antibody followed by Western blot analysis (Fig. 7B). Proteins isolated from nontransfected cells (Fig. 7B, lanes 1) or Tat-transfected cells (lanes 2) were used as negative and positive controls, respectively. The level of detected Tat slightly decreased in extracts prepared from Tat/p300-transfected cells (Fig. 7B, lane 3). Cell extracts were immunoprecipitated with polyclonal anti-p300 antibody (Fig. 7B, lanes 4 to 5) or anti-p73 antibody (lane 6), with rabbit serum (lane 7), or with Sepharose beads (lane 8). Western blot analysis of the immune complexes confirmed the existence of a direct association between Tat and p300 in the absence and the presence of p73 (Fig. 7B, lanes 4, 5, and 6, respectively).

Since Tat has the ability to interact with cyclin T1 through its N-terminal domain, we sought to study whether competition to interact with Tat exists between p73 and cyclin T1. As shown in Fig. 7C, protein from transfected cells was subjected to immunoprecipitation with a polyclonal anti-Tat or anti-p73 antibody followed by Western blot (we used anti-p73α C-17, which recognizes the C-terminal domain of p73α only [purchased from Santa Cruz Biotechnology]). Protein extracts from nontrans-

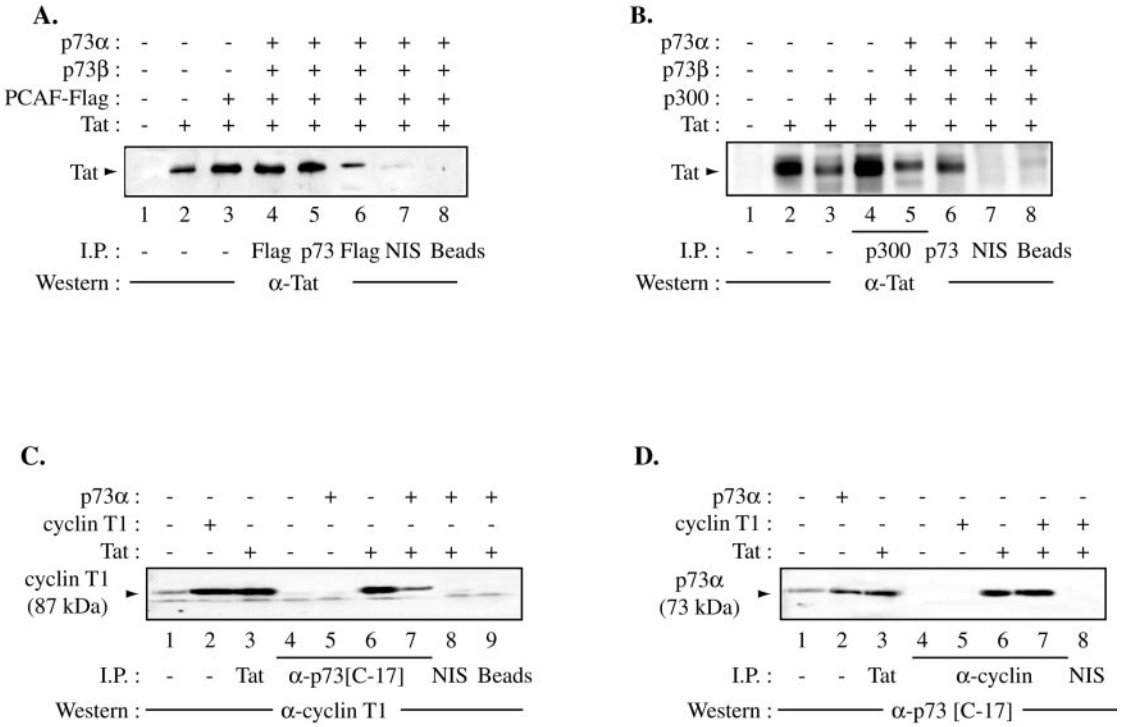

FIG. 7. Physical interaction between Tat/p73 and PCAF or cyclin T1. Cell lysates were prepared from U-87MG cells transfected with plasmids expressing different cDNA constructs as indicated above each panel. Approximately 300 μg of cell extract was utilized in immunoprecipitations (I.P.) with anti-Flag, anti-Tat, anti-p73, anti-cyclin T1, and anti-p300 antibodies or with rabbit preimmune serum (nuclear import signal [NIS]) (A and D, lanes 7, and B and C, lanes 8) or Sepharose beads (A and D, lanes 8, and B and C, lanes 9). Western analysis was performed using anti-Tat, anti-p73, and anti-cyclin T1 antibodies. In parallel, 50 μg of extracts was utilized by direct Western blot assay (A and B, lanes 1 to 3, and C and D, lanes 1 and 2). The arrow depicts the position of the detected proteins.

fected (Fig. 7C, lanes 1) or cyclin T1-transfected (lanes 2) cells were used as negative and positive controls, respectively. Cell extracts were immunoprecipitated with polyclonal anti-Tat antibody (Fig. 7C, lane 3) or anti-p73 antibody (lanes 4 to 7), with rabbit serum (lane 8), or with Sepharose beads (lane 9). Western blot analysis of the immune complexes confirmed the existence of a direct association between Tat and cyclin T1 in the absence of p73 (Fig. 7C, lane 3). Endogenous or overexpressed p73α failed to interact with cyclin T1 in the absence of Tat (Fig. 7C, lanes 4 and 5). Interestingly, in the presence of Tat, endogenous p73α interacts with cyclin T1 (Fig. 7C, lane 6). When p73α was overexpressed in the presence of Tat, a very weak association between p73α and cyclin T1 was observed (Fig. 7C, lane 7).

Reciprocally, as shown in Fig. 7D, proteins isolated from nontransfected (lanes 1) or p73α-transfected (lanes 2) cells were used as negative and positive controls, respectively. Cell extracts were immunoprecipitated with polyclonal anti-Tat antibody (Fig. 7D, lane 3) or anti-cyclin T1 antibody (lanes 4 to 7) or with rabbit serum (lane 8). Western blot analysis of the immune complexes confirmed the existence of a direct association between Tat and p73α in the absence of p73 (Fig. 7D, lane 3). Endogenous or overexpressed cyclin T1 failed to interact with p73α in the absence of Tat (Fig. 7D, lanes 4 and 5). Interestingly, in the presence of Tat, endogenous cyclin T1 interacts with p73α (Fig. 7D, lane 6). When cyclin T1 was overexpressed in the presence of Tat, a very strong association between p73α and cyclin T1 was observed (Fig. 7D, lane 7).

p73 prevents interaction between cyclin T1 and Tat in vitro. The ability of cyclin T1 and p73 to associate in the presence of Tat led us to investigate whether this interaction is direct or mediated through a third factor. GST pull-down assays were performed using ^{35}S IVT p73α, p73β, cyclin T1, ΔN-p73α, and ΔN-p73β. The translated proteins were incubated with GST, GST-Tat, or GST-Tat(Δ2–36). As shown in Fig. 8, p73α, p73β, and cyclin T1 interact with GST-Tat (lanes 3) but not with GST alone (lanes 2) or GST-Tat(Δ2–36) (lanes 4). Interestingly, cyclin T1 failed to interact with GST-Tat (Fig. 8, lanes 3) when incubated in the presence of p73α or p73β but not in the presence of ΔN-p73α or ΔN-p73β. As expected, ΔN-p73α and ΔN-p73β were incapable of interacting with GST-Tat either alone or in the presence of cyclin T1.

Subcellular distribution of p73 in the presence of Tat. Since there is a physical and functional interplay between Tat and p73, this led us to determine their subcellular localization. U-87MG cells were transfected with CFP, CFP-Tat, and pDsRed1-mito expression plasmids. The levels of Tat and/or p73 production and their subcellular localization were assessed by immunocytochemistry. As shown in Fig. 9, at 48 h, cells transfected with CFP-Tat exhibited exclusive nuclear localization, while a vector expressing CFP alone showed only cytoplasmic localization. Similar to Tat, at 48 h, in Tat-transfected cells, endogenous p73 also localized to the nucleus, while it partially localized to the cytoplasm in the absence of Tat.

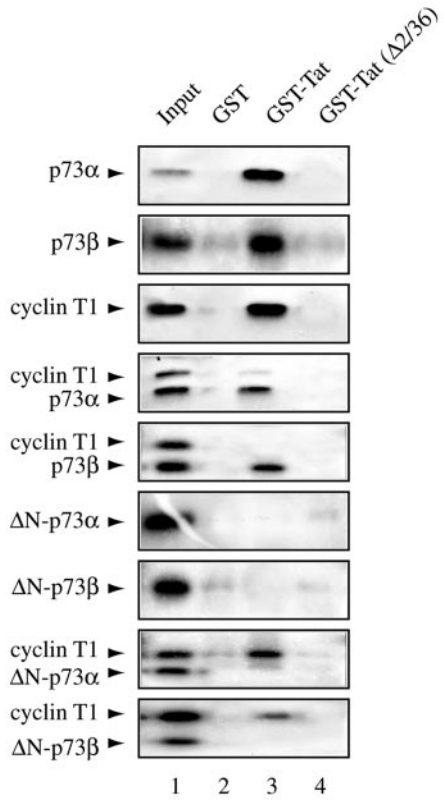

FIG. 8. In vitro interaction between Tat and p73 in the presence of cyclin T1. Wild-type Tat or the indicated mutants fused to GST and immobilized on glutathione-Sepharose were incubated with in vitro-synthesized [^{35}S]methionine-labeled p73α, p73β, ΔN-p73α, ΔN-p73β, or cyclin T1 proteins in various combinations. After incubation for 2 h at 4°C, the bound proteins were eluted and analyzed by SDS-PAGE.

These observations confirmed that p73 colocalizes with Tat in these cells.

DISCUSSION

The HIV-1 Tat protein is a key pathogenic factor in a variety of AIDS-associated disorders. We provided experimental evidence indicating that HIV-1 Tat binds directly to the potential tumor suppressor p73. We confirmed the interaction between Tat and p73 by IP followed by Western blot (IP/Western) and GST pull-down assays. The use of recombinant proteins allowed us to demonstrate that Tat binds directly to the N-terminal domain of p73. Most importantly, the basic function of Tat was significantly altered when the two proteins interacted. RNA interference using p73 siRNA confirmed the functional importance of the Tat/p73 interaction on HIV-1 LTR transcription in vivo. The physical presence of p73 and Tat at the HIV-1 LTR was directly demonstrated by ChIP assays. Association of p73 with Tat inhibits acetylation of lysine 28 of Tat without preventing interaction of Tat with either PCAF or cyclin T1. These findings provide functional evidence that Tat and p73 interact in infected cells and that this interaction may be biologically relevant in regard to Tat-mediated AIDS pathogenesis.

Identification of the Tat-interacting domain of p73. Unlike p53 which was originally demonstrated to bind to the C-terminal region of Tat (3), the cysteine-rich domain of Tat binds to the N-terminal domain of p73, and loss of the binding results in induction of Tat-mediated apoptosis. Furthermore, unlike p53, whose N-terminal domain (aa 1 to 45) is not absolutely essential for its apoptotic function (42), p73 appears to require its N terminus for apoptosis. This suggests that the transactivation function of p73 may be closely related to its apoptotic function. It is interesting that p73 and p53 use different domains to target distinct domains of Tat. One explanation could be that although p73 and p53 share an overall 29% identity in amino acid sequence within their N termini (23), the p73 N terminus is 16 residues longer than that of p53. This additional sequence in the center of the p73 N terminus may cause conformational modification that could in turn influence the manner by which p73 physically interacts with Tat. It is possible that the difference between p73 and p53 may serve to avoid a direct competition for the same set of proteins. Furthermore, it has been shown that p73 has the ability to bind other proteins through its N-terminal domain and that the interaction of this domain with Tat is not without a precedent. It has been shown that p73 uses its N-terminal domain to bind to cellular coactivators p300 and CBP, facilitating apoptosis (54). Therefore, through its binding to the N-terminal domain, Tat might neutralize p73's proapoptotic function in its interaction with p300, since the same N-terminal domain is utilized by p73 to bind p300 (54). Inhibition of p73 through its N terminus by viral proteins has also been described with human papillomavirus types 11 and 16, in which the E6 protein was shown to physically interact with and inactivate p73 (33).

In addition to its nuclear localization, the p73 protein was also shown to adopt a cytoplasmic localization. It is very important to understand and to identify the mechanisms used by p73 to adopt the same subcellular localization in the presence of Tat. Furthermore, we are in the process of studying whether/how Tat affects the nuclear import as well as the nuclear export of p73.

Identification of the p73-interacting domain of Tat. The cysteine-rich region of Tat has been shown to be involved in its ability to cause cell death by apoptosis (53). This observation was also confirmed by other studies in which the cysteine-rich domain of Tat was shown to be involved in apoptotic death of endothelial cells (19) and in the induction of tumor necrosis factor-related apoptosis-induced ligand, which leads to cell death (51). In addition, through its cysteine-rich domain, Tat has the ability to bind several proteins involved in acetylation, including the coactivator p300, PCAF, and hGCN5 (6, 31, 47). The Tat protein is acetylated on lysine residues 28 and 50 within its TAR RNA-binding arginine-rich motif by the transcriptional coactivators p300/CBP and PCAF, respectively (8, 22, 24). Acetylation of Tat on K28 by PCAF facilitates recruitment of the P-TEFb (cyclin T1-cdk9) complex to the HIV-1 TAR RNA and dissociates Tat-PCAF interactions. Acetylation of lysine 50 by p300/CBP prevents binding of acetylated Tat to TAR RNA (5, 24). Acetylation of lysine 50 also creates a new binding site for PCAF and facilitates the formation of a PCAF–Tat–PTEF-b complex (5). The bromodomain of PCAF specifically binds acetylated lysine 50, and this interaction has been demonstrated to prevent K50-acetylated Tat from bind-

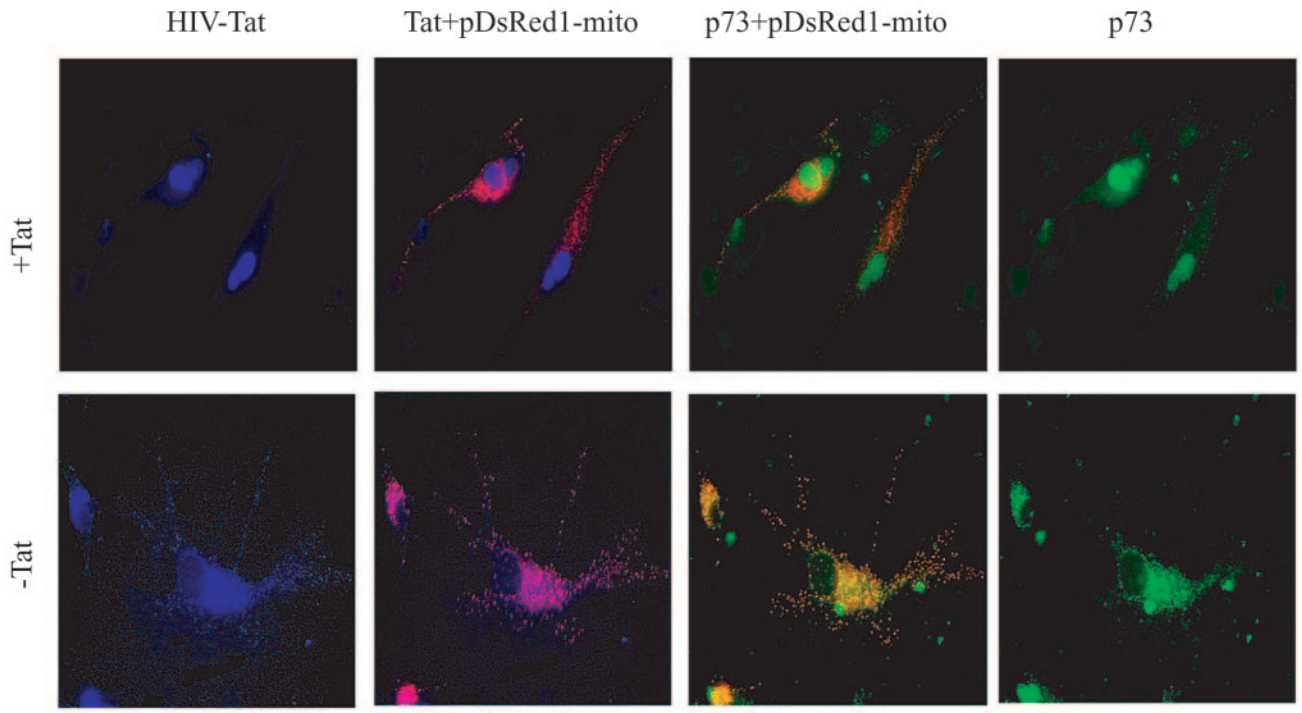

FIG. 9. Subcellular localization of Tat and/or p73. CFP-Tat-transfected cells demonstrate the nuclear subcellular localization of the HIV transactivator protein (blue); in these Tat positive cells, p73 is also localized with the same nuclear distribution (green). In cell cultures transfected with a CFP-tagged empty vector, the location of the vector is exclusively cytoplasmic (−Tat panel, blue). The DsRed1 plasmid (red) was used to mark the mitochondria. Immunolabeling shows the nuclear localization of Tat or p73 (Tat+mito and p73+mito panels). For all panels, original magnification is ×400.

ing TAR RNA (5, 31). This observation may explain our results in which PCAF retained its ability to interact with Tat in the presence of p73 because it interacts with lysine 50. This may also explain the ability of cyclin T1 to associate with p73 in the presence of Tat, due to its interaction with PCAF. Furthermore, recruitment of p300/CBP and PCAF to the HIV-1 LTR by Tat is essential for viral replication and activation of HIV-1 gene expression (5, 17, 20). This result might also explain the weak activation of HIV-1 LTR by Tat in the presence of p73 (Fig. 4A), which could be due to the weak association of PCAF with Tat in the presence of p73.

Moreover, the fact that PCAF and p300/CBP are limiting in cells provides further support for a mechanism that involves competition with, or implication of, a third protein. The observation that Tat and p73 reciprocally repress transcription in the presence of a direct interaction strongly suggests competition for limiting p300/CBP or PCAF. In addition, the observation that p73 and cyclin T1 associate in vivo but not in vitro also suggests the presence of a third factor, which could be PCAF or another protein. Finally, and similar to our results, Lemasson and Nyborg (25) demonstrated the existence of a physical interaction followed by a competition between p73 and human T-cell leukemia virus type 1 (HTLV-1) Tax protein to bind p300. The competition between these two proteins led to transcriptional suppression.

Significance of the functional interplay between p73 and Tat. At the transcriptional level, p53 was shown to downregulate the activation of HIV-1 LTR in several cell lines including CNS cells through its physical interaction with NF-κB or Sp1 proteins (4, 9, 14, 36, 40). These results are in accord with the results described previously by Deb et al., where p73 was shown to downregulate HIV-1 LTR expression (7). However, our results demonstrated that p73 acts as an activator in astrocytic cells (Fig. 4A). Therefore, the effect of p73 on HIV-1 gene expression might be tissue specific. Furthermore, while the mechanisms used by p53 to modulate HIV-1 LTR expression are known, the mechanisms used by p73 remain to be identified. Furthermore, our results demonstrated that, similar to p53 (28), p73 is a potent suppressor of Tat. Reciprocally, Tat inhibits the transcription of p53 through downregulation of the p53 promoter (28), while in our hands, Tat activates the p73 promoter (data not shown). Finally, Tat impairs p53 function by preventing its acetylation in immune/neuron-derived cells, thus favoring the establishment of neoplasia during AIDS (15). These results are in accord with our results obtained from protein-protein interaction where Tat affects p73 function by preventing its acetylation in U-87MG cells (data not shown). Furthermore, our results clearly showed that endogenous p73 has a negative effect on HIV LTR expression that is removed when endogenous p73 is downregulated.

Relevance of Tat-p73 interaction on HIV-1 pathogenesis. The p73 gene is monoallelically expressed in normal tissues, maps to chromosome 1p36, and is deleted in human neuroblastoma cell lines. p73's classification as a tumor suppressor protein remains controversial, and given that p73 is rarely mutated in human tumors, it is unlikely that p73 acts as a tumor suppressor like p53. It has been shown that in astrocytic gliomas, p73 does not play a major role as a tumor suppressor

(1). Furthermore, identification of a new p73 (ΔN-p73) isoform lacking the transactivation domain provides an alternative role for p73 during neuronal development, suggesting that ΔN-p73 is protective against apoptosis (34). The interaction of Tat with the N-terminal region of p73 may allow p73 to act like the *trans*-dominant mutant ΔN-p73 and therefore might prevent Tat-induced apoptosis. Interestingly, ΔN-p73 failed to interact with Tat and failed to prevent Tat's acetylation. These results demonstrated the necessity of Tat to associate with the N-terminal domain of p73 in order to be inactive or to inhibit the functions of p73. This scenario remains to be investigated.

Furthermore, p53 has recently been shown to participate in the development of HIV-associated dementia (HAD) in patients (12). Accumulation/overexpression of p53 protein in neurons was reported in postmortem cortical tissue from a small group of HAD patients (12, 38). Using a much larger cohort of HAD cases, it has been reported that p53 protein also increases in nonneuronal cells, including microglia and astrocytes (41, 43). These results are in accord with our results obtained with p73, where we showed using gene microarray and immunohistochemistry techniques that the endogenous p73 level is elevated in the brain of HIV-1-infected patients (data not shown).

Recently, we demonstrated that p73 negatively affects Tat-induced apoptosis in neural cells (data not shown). Taken together with the data presented in this paper, we suggest that the biological relevance of p73-Tat interaction where both proteins affect each other's functions can be explained as a self-defense mechanism used by the neural cells to protect against HIV-1 replication. Similar mechanisms were observed for p53 (9, 12, 28, 29). Therefore, molecular manipulation leading to the induction of p73 in HIV-1-infected cells could represent a novel therapeutic approach against Tat-induced neuropathogenesis, which occurs in AIDS patients.

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