Human Immunodeficiency Virus Type-1 Tat/Co-activator Acetyltransferase Interactions Inhibit p53<sup>Lys-320</sup> Acetylation and p53-responsive Transcription

Patients with AIDS are at increased risk for developing various neoplasms, including Hodgkin's and non-Hodgkin's lymphomas, Kaposi's sarcomas, and anal-rectal carcinomas, suggestive that human immunodeficiency virus type-1 infection might promote establishment of AIDS-related cancers. Tat, the viral trans-activator, can be endocytosed by uninfected cells and has been shown to inhibit p53 functions, providing a candidate mechanism through which the human immunodeficiency virus type-1 might contribute to malignant transformation. Because Tat has been shown to interact with histone acetyltransferase domains of p300/cAMP-responsive element-binding protein (CREB)-binding protein and p300/CREB-binding protein-associated factor, we have investigated whether Tat might alter p53 acetylation and tumor suppressor-responsive transcription. Here, we demonstrated that both Tat and p53 co-localize with p300/CREB-binding protein-associated factor and p300 in nuclei of IMR-32 human neuroblastoma cells and in PC-12 pheochromocytoma cells. Further, p53 <i>trans</i>-activation of the 14-3-3<i>ζ</i> promoter was markedly repressed by Tat histone acetyltransferase interactions, and p53 acetylation by p300/CREB-binding protein-associated factor on residue Lys<sup>320</sup> was diminished as a result of Tat-histone acetyltransferase binding <i>in vivo</i> and <i>in vitro</i>. Tat also inhibited p53 acetylation by p300 in a dosage-dependent manner <i>in vitro</i>. Finally, HIV-1-infected Molt-4 cells displayed reduced p53 acetylation on lysines 320 and 373 in response to UV irradiation. Our results allude to a mechanism whereby the human immunodeficiency virus type-1 <i>trans</i>-activator might impair tumor suppressor functions in immune/neuronal-derived cells, thus favoring the establishment of neoplasia during AIDS.

Although the most frequent malignancies observed in AIDS patients are non-Hodgkin's lymphomas, central nervous system non-Hodgkin's lymphomas, and Kaposi's sarcomas, compartmentalization of human immunodeficiency virus, type-1 (HIV-1)<sup>1</sup> in the central nervous system might be associated with the recent increase of rare intracranial tumors, such as glioblastomas, anaplastic astrocytomas, and subependymomas (1–8). The viral <i>trans</i>-activator, Tat, can be endocytosed by surrounding uninfected cells and has been demonstrated to inhibit the G<sub>2</sub>/S arrest-inducing functions of p53, providing a candidate mechanism through which HIV-1 might contribute to malignant transformation in the central nervous system (4–7). Tat is a 82–101-amino acid peptide that contains an arginine-rich motif required for binding a uracil-containing bulge in the Tat-associated region (TAR) of HIV-1 transcripts (8). Interactions between Tat/TAR-RNA stabilize viral mRNAs; thus, Tat principally acts as an elongation factor to enhance long terminal repeat <i>trans</i>-activation. The arginine-rich motif of Tat interacts with the catalytic histone acetyltransferase (HAT) domains of transcriptional co-activators, p300/CREB-binding protein (CBP) and p300/CBP-associated factor (P/CAF);HGCN5 (9–15), and Tat is acetylated by p300 on lysine residues Lys<sup>50</sup>/Lys<sup>51</sup> (Lys<sup>51</sup> is only weakly acetylated) and by P/CAF on Lys<sup>28</sup> (12, 16–18). Acetylation of Tat on Lys<sup>50</sup> diminishes its binding affinity for TAR-RNA, and acetylation on Lys<sup>28</sup> enhances Tat binding to the Tat-associated kinase complex containing cdk9 and human cyclin T1 (16, 17). Importantly, Mutjaba <i>et al. (19)</i> demonstrated that acetylated Tat peptide interacts with the bromodomain of P/CAF, and this interaction could play an important role in dissociation of Tat/TAR-RNA complexes (20–22). Tat/co-activator interactions are essential for HIV-1 long terminal repeat <i>trans</i>-activation (9, 12–18). Because the HAT domains of p300/CBP and P/CAF also target p53 for acetylation on residues Lys<sup>375</sup>/Lys<sup>372</sup> and Lys<sup>320</sup>, respectively, we hypothesized that Tat-HAT interactions might competitively interfere with p53 acetylation and, consequently, tumor suppressor-responsive transcription functions (23–25).

**MATERIALS AND METHODS**

<i>Cell Culture, Immunofluorescence Laser Confocal Microscopy, and FACS—IMR-32 human neuroblastoma cells (ATCC number CCL-127)</i>

<i>1</i> The abbreviations used are: HIV-1, human immunodeficiency virus, type-1; TAR, Tat-associated region; CBP, CREB-binding protein; P/CAF, p300/CBP-associated factor; HAT, histone acetyltransferase; CREB, cAMP-responsive element-binding protein; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; GST, glutathione S-transferase; CMV, cytomegalovirus; RSV, Rous sarcoma virus.
were cultured in ATCC 2003, Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin-sulfate (Invitrogen). PC-12 rodent pheochromocytoma cells (ATCC number CRL-1721) were cultured in ATCC F-12K medium supplemented with 5% fetal bovine serum, 15% horse serum, 100 units/ml penicillin, and 100 µg/ml streptomycin-sulfate. All of the cells were grown either in tissue culture dishes or eight-chamber slides (Nalge Nunc International) coated with mouse type IV collagen (Invitrogen) and were incubated under 10% CO2 at 37 °C. The transfections were performed using a calcium-phosphate system as recommended by the manufacturer (Invitrogen). Calu-6 carcinoma cells (ATCC number HTB-56) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin-sulfate and were transfected using LipofectAMINE reagent (Invitrogen) as recommended in the manufacturer's protocol. Molt-4 CD4⁺ lymphoblastoid leukemia cells were grown at 37 °C under 10% CO2 in RPMI medium supplemented with 20% human donor serum (Sigma), 100 units/ml penicillin, and 100 µg/ml streptomycin-sulfate. The protein extracts were prepared by rapid freeze-thawing followed by centrifugation at 14,000 rpm at 4 °C and quantified using the Bradford microassay and spectrophotometric analyses at 595 nm; 20 µl from each sample was measured using a luciferase assay kit (Promega Corp.) and a Lumat model LB 9501 luminometer (Berthold, Inc.). All of the experiments were carried out as dose responses in two different cell-lines (IMR-32 and PC-12) or in duplicates for single-point analyses (error bars representative of standard deviations are shown). Immunofluorescence laser confocal microscopy was performed on IMR-32, PC-12, or HIV-1-infected Molt-4 cells using a Leica TCS spectrophotometric confocal microscope equipped with krypton and argon lasers, controlled by a Windows NT-based work station. Relative fluorescence intensities were quantified using TCS linear quantification software. Briefly, the cells were fixed in 0.2% glutaraldehyde and 1% formaldehyde in PBS, and nonspecific antigens were blocked by incubation with 3% (w/v) bovine serum albumin, 0.5% (v/v) Tween 20 in PBS. HIV-1 Tat was detected using the rabbit primary antibody, C-2145, and p35 was detected using either a monoclonal anti-p53 antibody (BP53, Upstate Biotechnology, Inc.) or an anti-p53 rabbit polyclonal antibody (Santa Cruz Biotechnologies, Inc.). p300 was detected using a monoclonal anti-p300 CT antibody (RW-128; Upstate Biotechnology, Inc.) or a rabbit polyclonal antibody (N-15; Santa Cruz Biotechnologies, Inc.). P/CAF was detected using a goat polyclonal antibody (C-16; Santa Cruz Biotechnology, Inc.). Fluorescent secondary antibodies were used in appropriate combinations: rhodamine red-conjugated donkey anti-mouse IgG, fluorescein isothiocyanate- or Cy-3-conjugated donkey anti-rabbit IgG, or rhodamine red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). For apoptosis and cell cycle analyses, transfected IMR-32 cells were treated with an arrest-inducing concentration of adriamycin (100 µM) and incubated for 24 h at 37 °C. The cells were harvested, washed twice with PBS, and then resuspended in 1× annexin-buffer and stained for annexin-V surface expression for 10 min (Pharminc, Inc.) or fixed in 3.7% formaldehyde/PBS for 10 min, washed, and permeabilized in 0.1% IGEFAL CA-630 (Sigma)/PBS containing 5 µg/ml RNase and incubated on ice for 15 min. The samples were then stained using acridine orange (Molecular Probes, Inc.) at 4 °C for 30 min. FACS analyses were performed using a Becton Dickinson, FACSCalibur flow cytometer. The cell cycle analyses were gated to exclude aggregates and fragments.

**Protein Purification and In Vitro Acetylation—GST-Tat, GST-TatK28A/K50A, and GST-p53 fusion proteins were expressed in Escherichia coli; strain DH5α, by induction with 100 µM isopropyl-β-D-thio-galactopyranoside (Invitrogen) in LB broth containing 100 µg/ml ampicillin at 37 °C overnight. The cells were pelleted at 5000 rpm, washed twice with PBS, centrifuged, and resuspended in 5 ml of cold PBS containing the protease inhibitors antipain-dihydrochloride, bestatin, chymostatin, leupeptin, and pepstatin (Roche Molecular Biochemicals) at 50 µg/ml each. Bacteria were lysed by sonication over an ice bath, and the sonic dissmenm matrices (Invitrogen) were centrifuged at 2500 rpm at 4 °C and then washed twice with PBS (with protease inhibitors). Bound GST fusion proteins were eluted in four 200-µl fractions of 10 mM reduced glutathione (Sigma) in PBS (with protease inhibitors). The eluted fractions were analyzed by electrophoresis through a 12.5% SDS-polyacrylamide gel; the proteins were visualized by Coomassie staining. Purified proteins were dialyzed overnight at 4 °C against 25 mM Heps, pH 7.9, 5 mM KCl, 0.5 mM MgCl2, 0.5 mM EDTA, 0.25 mM dithiothreitol, and 10% glycerol; the fractions were stored in 20-µl aliquots at −80 °C. For in vitro acetylation, 100 ng of purified GST-p53 was mixed either with 5 units of purified p300 or

![Fig. 1. HIV-1 Tat inhibits p53-responsive transcription function.](image-url)
FIG. 2. HIV-1 Tat and adenoviral E1A 12S proteins repress p53-responsive transcription to similar degrees. A, IMR-32 cells were co-transfected with expression constructs for Tat acetylation-defective mutants (3 μg), RSV-Tat, RSV-TatK28A, and RSV-TatK28A/K50A, and 1 μg of CMV-p53g (or CMV-p53R175H) with 1 μg of 14-3-3ζ luciferase. The protein levels are shown for p53, p53R175H, Tat, TatK28A, TatK28A/K50A, and actin for extracts (50 μg of total proteins) used in A, B, and C, PC-12 cells (B) and IMR-32 cells (C) were co-transfected with 14-3-3ζ luciferase and CMV-p53 in the presence of increasing amounts of CMV-E1A 12S or CMV-E1A 12SAN (0.5, 1.5, or 3 μg). D, IMR-32 cells were co-transfected with 14-3-3ζ luciferase, CMV-p53g, and 3 μg of RSV-Tat, either in the presence of increasing amounts of CMV-P/CAF or CMV-p300 (1.5, 2.5, or 5 μg). WT, wild type.

P/CAF (Upstate Biotechnology, Inc.) in acetylation buffer containing 50 mM Tris-Cl, pH 8.0, 1 mM diithiothreitol, 0.1 mM EDTA, 50 μg/ml bovine serum albumin, 10% glycerol, and 5 μl of [acetyl-1-14C]acetyl coenzyme A (51.60 mCi/mmol, NEC-313; PerkinElmer Life Sciences) in the presence or absence of increasing concentrations of GST-Tat or GST-TatK28A/K50A (25, 50, or 100 ng) or Tat synthetic peptides (Tat₃₃₋₄₀, Tat₄₁₋₅₀, or Tat₅₁₋₆₀) with 1 μg of 14-3-3ζ luciferase, CMV-p53g, and 1 μg of CMV-P/CAF (Upstate Biotechnology, Inc.). Tat expression was measured in lysates prepared from infected cells by immunoblotting using a rabbit polyclonal antibody that specifically recognizes Tat protein determined by a standard anti-p24gag enzyme-linked immunosorbent assay method. Aliquots were taken from HIV-1-infected samples on consecutive days (4–6 days post-infection), and half of the volume of infected cells were UV-irradiated for 1.5 min (Fisher UV-Crosslinker at 120 mJ/cm² energy level), before culturing for an additional 3 h at 37°C under 10% CO₂. Induction of p53 protein by UV treatment was confirmed by immunofluorescence laser confocal microscopy and immunoblotting using uninfected or HIV-1-infected cells and a p53-specific monoclonal antibody (Upstate Biotechnology, Inc.). Tat expression was measured in lysates prepared from infected cells by immunoblotting using a rabbit polyclonal anti-Tat antibody or by direct visualization by immunofluorescence laser confocal microscopy. Co-localization of Tat and p53 in UV-irradiated cells was determined and quantified by immunofluorescence laser confocal microscopy. The status of p53 acetylation on lysine residues Lys³²⁰ and Lys³⁷⁷ was detected by immunoprecipitating total intracellular p53 from untreated or UV-irradiated, HIV-infected Molt-4 cells. The protein G-agarose immune complexes were washed, and the bound products were resolved through 4–20% Tris-glycine gels (Invitrogen, Inc.) and visualized by autoradiography/fluorography using ENHANCE reagent (PerkinElmer Life Sciences). Kodak XAR scientific imaging film was exposed for 72 h at ~80°C.

Effects of HIV-1 Infection upon UV-responsive p53 Acetylation—To assess effects of HIV-1 infection upon p53 acetylation in response to UV irradiation in vivo, we infected 3 × 10⁶ Molt-4 CD4⁺ lymphoblastic leukemia cells with 150 μl of an infectious stock of HIV-1, HXB₂m (HXB₂m virus stock and Molt-4 cells were generously provided by Dr. G. Rodriguez, Food and Drug Administration, Center for Biologics Evaluation and Research, National Institutes of Health, Bethesda, MD), titrated at ~2 × 10⁶ pg/ml of p24gag protein determined by a standard anti-p24gag enzyme-linked immunosorbent assay method. Aliquots were taken from HIV-1-infected samples on consecutive days (4–6 days post-infection), and half of the volume of infected cells were UV-irradiated for 1.5 min (Fisher UV-Crosslinker at 120 mJ/cm² energy level), before culturing for an additional 3 h at 37°C under 10% CO₂. Induction of p53 protein by UV treatment was confirmed by immunofluorescence laser confocal microscopy and immunoblotting using uninfected or HIV-1-infected cells and a p53-specific monoclonal antibody (Upstate Biotechnology, Inc.). Tat expression was measured in lysates prepared from infected cells by immunoblotting using a rabbit polyclonal anti-Tat antibody or by direct visualization by immunofluorescence laser confocal microscopy. Co-localization of Tat and p53 in UV-irradiated cells was determined and quantified by immunofluorescence laser confocal microscopy. The status of p53 acetylation on lysine residues Lys³²⁰ and Lys³⁷⁷ was detected by immunoprecipitating total intracellular p53 from untreated or UV-irradiated, HIV-infected Molt-4 cells. The protein G-agarose immune complexes were washed, and the bound products were resolved through 4–20% Tris-glycine gels (Invitrogen, Inc.) and visualized by autoradiography/fluorography using ENHANCE reagent (PerkinElmer Life Sciences). Kodak XAR scientific imaging film was exposed for 72 h at ~80°C.

RESULTS

Inhibition of p53-dependent Transcriptional Activation by HIV-1 Tat—We first assayed whether Tat-HAT interactions might interfere with neuronal p53-responsive transcription in human IMR-32 neuroblastoma cells and rodent PC-12 pheochromocytoma cells. Because p53 regulates G₂/M cellular arrest by driving 14-3-3ζ promoter transactivation, we investigated the effects of HIV-1 Tat expression upon a 14-3-3ζ promoter luciferase reporter construct containing three p53-responsive elements (other regulatory elements are deleted in this plasmid. Refs. 26 and 27). As shown in Fig. 1, HIV-1 Tat effectively inhibited p53 transactivation from the 14-3-3ζ promoter in IMR-32 and PC-12 cells, respectively, whereas neither the TatK28A/K50A mutant nor green fluorescent protein significantly influenced p53 transcription. Consistently, Tat expression did not seem to alter p53 protein levels, suggesting that Tat causes functional impairment of p53 (Fig. 1A).

Ectopic P/CAF and p300 Counter Inhibitory Effects of HIV-1 Tat upon 14-3-3ζ Promoter trans-Activation—To evaluate the relative contributions of Tat-p300 or Tat-P/CAF interactions toward the inhibition of p53 transcription, we analyzed a panel
of previously characterized Tat mutants; the Tat<sub>K28A/K50A</sub> mutant is defective for acetylation by P/Caf, and Tat<sub>K50A</sub> is impaired for their abilities to repress p53-responsive 14-3-3<sub>H9268</sub>/H9262 and GST fusion proteins (GST-p53, GST-Tat, and GST-Tat<sub>K28A/K50A</sub>) were detected using a goat anti-GST antibody (Amersham Biosciences). HIV-1 Tat, GST-Tat, and GST-Tat<sub>K28A/K50A</sub> were expressed as described under "Materials and Methods." GST fusion proteins (GST-p53, GST-Tat, and GST-Tat<sub>K28A/K50A</sub>) were detected using a goat anti-GST antibody (Amersham Biosciences).

**Fig. 3.** Nuclear expression of p53, HIV-1 Tat, and Tat<sub>K28A/K50A</sub> in transfected IMR-32 cells. IMR-32 neuroblastoma cells were transfected with 0.5 μg of CMV-p53 (A), RSV-HIV-1 Tat (B), or RSV-Tat<sub>K28A/K50A</sub> (C) and immunostained for laser confocal microscopy using a rabbit polyclonal antibody against p53 (Santa Cruz Biotechnologies, Inc.) and fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. HIV-1 Tat and Tat<sub>K28A/K50A</sub> proteins were visualized using an anti-HIV-1-IMR Tat, rabbit polyclonal antibody (C-2145), and fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. Endogenous p500 was detected using a monoclonal antibody against the carboxyl terminus of p500 (Upstate Biotechnology, Inc.) and rhodamine red-conjugated anti-mouse secondary antibody. Endogenous P/Caf was detected using a goat polyclonal antibody against P/Caf (Santa Cruz Biotechnologies, Inc.) and Cy-3-conjugated anti-goat secondary antibody. All of the fluorescence-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. The nuclei were stained with DAPI (Molecular Probes, Inc.) and are shown for reference. The merged images are at the right of each panel. Immunofluorescence laser confocal microscopy was performed using a 63× objective in combination with 15× digital zoom.

**Fig. 4.** Tat inhibits p53 acetylation in vivo and in vitro. A, a diagram of CBP (p300 synologue) and P/Caf depicting functionally overlapping binding sites for HIV-1 Tat, EIA 12S, and p53 in acetyltransferase domains. B, IMR-32 cells were co-transfected with 1 μg of CMV-p53 and 3 μg of RSV-Tat, or various Tat mutants (RSV-Tat<sub>K28A/K50A</sub>, RSV-Tat<sub>K28A</sub>, RSV-Tat<sub>K50A</sub>, or RSV-Tat<sub>K28A/K50A</sub>) extracts were prepared in RIPA buffer, and immunoblotting was performed using 30 μg of total proteins to detect p53, acetyl-p53<sub>Lys-320</sub>, acetyl-p53<sub>Lys-373</sub>, and actin. C, nuclear extracts were prepared from 3 × 10<sup>6</sup> IMR-32 cells co-transfected with 1 μg of CMV-p53 and either 3 μg of RSV-Tat, RSV-Tat<sub>K28A/K50A</sub>, RSV-Tat<sub>K28A/K50A</sub>, or RSV-Tat<sub>K28A/K50A</sub> and used to detect binding to 5′-radiolabeled, consensus cyclic AMP-responsive element (5′-CTTAGCATCGAT-GACGCAATACGCCATGGTCGA-3′) and p53-responsive (5′-TACA- GAACATGTCUTAGACATTGCGTTCAG-3′) oligonucleotide probes. D, amino acid sequences of wild-type Tat and Tat-derived synthetic peptides (Tat<sub>_4-45</sub>, Tat<sub>_K/K4-45</sub>, Tat<sub>_K/K50</sub> and Tat<sub>_K28A/K50A</sub>) used in in vitro acetylation assays. E and F, acetylation of GST-p53 by recombinant P/Caf (E) or p300 (F) in the presence of increasing amounts of GST-Tat, GST-Tat<sub>K28A/K50A</sub> or Tat-derived peptides was performed as described under "Materials and Methods." GST fusion proteins (GST-p53, GST-Tat, and GST-Tat<sub>K28A/K50A</sub>) were detected using a goat anti-GST antibody (Amersham Biosciences).
in nuclei of transfected IMR-32 neuroblastoma cells, as determined by immunofluorescence laser confocal microscopy, and both proteins displayed varied levels of co-localization with the transcriptional co-activator/acetyltransferase p300 (Fig. 3, A and B). The subcellular distribution of Tat was observed to be both nucleoplasmic and nuclear in IMR-32 cells, as revealed by immunofluorescence laser confocal microscopy (Fig. 3B). Importantly, the Tat-derived mutant, TatK28A/K50A, was present in the nuclei of IMR-32 cells (Fig. 3C). We examined the localization of TatK28A/K50A because the K50A mutation is targeted within the nuclear localization sequence of Tat, and it was therefore necessary to establish whether the mutant protein is properly expressed in the nuclei. In addition, we observed that nuclear P/CAF levels appeared to be somewhat limiting in IMR-32 neuroblastoma cells, as revealed by comparatively weak P/CAF-specific nuclear immunostaining (Fig. 3, A–C, lower panels).

**HIV-1 Tat and Tat Synthetic Peptides Inhibit p53 Acetylation by P/CAF and p300**—Direct interactions of co-activator HATs with Tat, p53, and E1A 12S provide a plausible basis for competitive inhibition of factor acetylation (Fig. 4A and Refs. 17, 23, 25, and 30). In IMR-32 cells co-transfected with p53 and either Tat or various Tat mutants, the wild-type Tat caused markedly diminished p53Lys-320 acetylation by P/CAF in vivo, whereas no significant difference was observed for p300-dependent, p53Lys-373 acetylation (Fig. 4B). The Tat-derived mutants did not affect p53 acetylation, despite having partially repressive effects upon p53-responsive transcription. These discrepancies may derive from weakened co-activator-binding affinities for the mutants or could suggest that alternate signaling interactions contribute to Tat-repression of p53 transcription. Overexpression of p53 did not result in increased levels of p53-acetylated forms (compare lanes 1 and 2 of Fig. 4B), supporting reports that nuclear co-activator concentrations are limiting (32, 33). That no apparent difference was observed for p300-dependent, p53Lys-373 acetylation suggests that the restoring effect of ectopic p300 upon 14-3-3 peptide affinity matrices was washed, the samples were electrophoresed through a 4–20% SDS-PAGE precast gel (Invitrogen, Inc.), and bound HIV-1 Tat was detected using purified anti-HIV-1 Tat polyclonal antibody. B. p53-null, Calu-6 carcinoma cells were transfected with 3 µg of CMV-p53 or CMV-vector control, the extracts were prepared in RIPA buffer, and equivalent amounts of total cellular proteins were used in in vitro binding assays. Increasing amounts of synthetic HIV-1 TatK23–43 or TatK23–61 peptides (250 ng, 500 ng, and 1 µg) were mixed with Calu-6 extracts expressing p53, and 15 units of purified recombinant GST-P/CAF(352–432) were added to each sample. 30 µl of 50% glutathione-Sepharose 4B in PBS was added to each reaction, and GST-pull-down assays were carried out overnight at 4 °C. Affinity matrices were washed twice with PBS, the samples were resolved by 12% SDS-PAGE, and bound p53 was detected using an anti-p53 monoclonal antibody. Input levels of synthetic TatK23–43 and TatK23–61 peptides were detected by electrophoresis through a 4–20% SDS-PAGE precast gel and Coomassie staining.
**FIG. 6. HIV-1 Tat causes bypass of G2/M arrest induced by adriamycin.**

A. IMR-32 cells were transfected with RSV-Tat, RSV-TatK28A/K50A, RSV-TatK28A, or RSV-TatK50A, and certain samples were treated with 100 μM adriamycin (Adr) and stained for annexin-V surface expression.

B. Overlays of FACS profiles for annexin-V staining in the presence of adriamycin and either wild-type (WT) Tat or TatK28A/K50A.

C. IMR-32 cells were transfected and treated with adriamycin as in A and were stained to determine nuclear contents using acridine orange as described under "Materials and Methods."
tion. To address this question, we expressed p53 in the background of p53-null, Calu-6 carcinoma cells and used equivalent amounts of total cellular proteins from prepared extracts in GST pull-down experiments. Increasing amounts of synthetic wild-type Tat23–43 or TatK28A23–43 mutant peptides were added to binding reactions in the presence of purified recombinant GST-P/CAF352–382, comprising the minimal acetyltransferase domain of P/CAF. As shown in Fig. 5B, the p53-P/CAF352–382 interaction was significantly diminished in a dosage-dependent manner in the presence of wild-type Tat23–43 peptide but was unaffected by the TatK28A23–43 mutant peptide. Indeed, because Tat23–43 peptides lack amino acid residues that are essential for p53 binding by Tat, we infer that HIV-1 Tat competes against p53 for binding to the minimal acetyltransferase domain of P/CAF in our in vitro assays.

Wild-type HIV-1 Tat, but Not TatK28A23–43, Facilitates Bypass of G2/M Cellular Arrest Induced by Adriamycin in IMR-32 Cells—Because 14-3-3 regulates p53-induced G2/M arrest under conditions of genotoxic stress, we addressed whether inhibition of p53 functions as a consequence of Tat-HAT binding might weaken the G2/M checkpoint in the presence of Adriamycin (100 μM; Refs. 26 and 27). IMR-32 cells were transfected with Tat or various Tat mutants; after 48 h, the cells were treated with Adriamycin and incubated for an additional 24 h prior to FACS analyses to evaluate apoptosis and cellular arrest using annexin-V (Pharmingen Corp.) and acridine orange (Molecular Probes, Inc.) staining methods. As shown in Fig. 6A, neither Adriamycin treatment nor Tat alone caused apoptosis in IMR-32 cells. However, cells expressing Tat in the presence of Adriamycin exhibited considerable programmed cell death (62.8%) as determined by annexin-V staining (Fig. 6, A and B). Of the Tat mutants, only TatK50A was associated with significant apoptosis (43.3%), suggesting that P/CAF-binding by Tat might be an important contributing factor for the bypass of G2/M arrest. The results from this “mitotic trap” assay suggest that Tat-expressing cells not arrested in G2/M enter mitosis (M phase) and are killed through the action of Adriamycin. Acridine orange staining/cell cycle analyses revealed that IMR-32 cells were arrested in G2/M (G1, 27.5%; G2/M, 47.0%) as a result of Adriamycin treatment (Fig. 6C). Expression of various Tat mutants had no effect upon cellular arrest in G2/M (G1, ~24%; G2/M, 50% for each). The wild-type Tat significantly prevented cellular arrest in the presence of Adriamycin (G1, 43.7%; G2/M, 34.4%) coincident with increased apoptosis (Fig. 6C; see also Fig. 6, A and B). A minor population of IMR-32 neuroblastoma cells that contained greater than G2/M DNA content were reproducibly detected in each sample, and these cells presumably possess inherent growth-regulatory defects resulting in deregulation of cytokinesis and S phase entry (Fig. 6). This aberrant cellular population did not appear to be affected by expression of HIV-1 Tat or Tat-derived mutants.

HIV-1 Infection Interferes with p53 Acetylation in Response to UV Irradiation—Finally, we examined the influence of HIV-1 infection upon acetylation of the p53 tumor suppressor in response to genotoxic stress caused by UV irradiation (24, 25, 34). CD4+ Molt-4 lymphoblastic leukemia cells were infected with HIV-1, HXB2, over a 6-day period, and consecutive aliquots were removed and exposed to UV irradiation. The cells were cultured for an additional 3 h before extracts were prepared for immunoprecipitation and immunoblot analyses. HIV-1 Tat protein expression was significantly detectable by the fourth day of infection and continued to increase through the sixth day (Fig. 7A). Ultraviolet irradiation is known to induce increased intracellular p53 levels (35, 36), and indeed, p53 expression was detectable in uninfected UV-treated cells (Fig. 7B, upper left panel). Uninfected control cells or HIV-1-
infected Molt-4 cells that were not exposed to UV irradiation did not contain high p53 protein levels as determined by immunofluorescence laser confocal microscopy (Fig. 1B, upper middle and right panels) and immunoblotting using an anti-p53 monoclonal antibody (Fig. 7D). Nuclear Tat and p53 co-localized in HIV-1-infected cells, consistent with reports by others that Tat directly interacts with the p53 tumor suppressor (6); relative fluorescence intensities and signal overlaps for both proteins were measured by linear quantification and are shown below the micrographs in Fig. 7C. To assess the status of p53 acetylation in response to UV irradiation in HIV-1-infected cells, we prepared whole cell extracts and examined the levels of intracellular p53 (Fig. 7D). Actin is shown as an input control. Immunoprecipitations were performed, and the acetylated forms of p53 were identified by immunoblotting using antibodies that discriminate between Lys$^{320}$-acetylated p53 or Lys$^{373}$-acetylated p53 (Fig. 7D). As demonstrated in Fig. 7D, there was slight diminishment of p53$^{Lys^{320}}$ acetylation in HIV-1-infected cells on the fifth day of infection in the absence of UV treatment. More apparent, however, was the decrease in both p53$^{Lys^{320}}$ and p53$^{Lys^{373}}$ acetylation induced by UV irradiation on the fifth and sixth days of HIV-1 infection. Interestingly, the observed reduction in UV-responsive p53 acetylation did not correlate with intracellular levels of Tat, suggesting that other virally encoded proteins or host factors might influence p53 acetylation and responses to genotoxic stress (Fig. 7A). These results indicate that HIV-1 infection significantly prevents acetylation of p53 by co-activators/acyltransferases in response to UV irradiation, and our in vitro evidence suggests that Tat could play an important role in mediating this inhibitory effect.

**DISCUSSION**

The tumor suppressor p53 induces the arrest of cell cycle progression in G1/S and G2/M under conditions of genotoxic stress (26, 27, 37, 38). Because AIDS-infected individuals demonstrate increased risks for developing various neoplasms, inhibition of p53$^{Lys^{320}}$ acetylation/transcription by Tat-HAT binding could promote the acquisition of deleterious mutations by subverting p53-regulated checkpoint defenses. p53 trans-activation functions and G2/M cell cycle control are regulated by post-translational modifications, e.g., acetylation and phosphorylation, and destabilization of p53 by Mdm2 is dependent upon p400/TRRAP-associated chromatin-remodeling complexes (39–46). Mdm2 and adeno viral E1A 12S and E1B proteins have been shown to inhibit p53 acetylation by P/CAF, and therefore, the P/CAF co-activator/acyltransferase may be a key modulator of p53 tumor suppressor-associated activities (47–49).

Recently, others have demonstrated that Tat inhibits acetyltransferase activities of the co-activators Tip60 and TAFII$_{250}$ thereby causing repression of cellular transcription (50, 51). Extracellular Tat produced by surrounding, infected cells might also enter and target p53 in nuclei of adjacent cells to thereby cause repression of cellular transcription (50, 51). Extracellular Tat produced by surrounding, infected cells might also enter and target p53 in nuclei of adjacent cells to thereby cause repression of cellular transcription. Interestingly, the majority of viral factors that interact with p300/CBP are derived from oncogenic viruses. Indeed, Tat has been demonstrated to transform primary B-cells and induce lymphomas in transgenic mice, suggesting that Tat might contribute to certain hematological malignancies (56). Seeo et al. (57) have reported that p300 and P/CAF acetyltransferases are inhibited by a cellular complex, INHAT (inhibitor of acetyltransferases), associated with the Set1/TAF-I$\beta$ oncoprotein involved in myeloid leukemia, indicative that inhibition of co-activator acetyltransferases might be generally linked to neoplastic transformation. Our results allude to a mechanism whereby HIV-1 Tat might impair tumor suppressor functions in immune/neuro-immune cells of the central nervous system, thus supporting the establishment of AIDS-related cancers.

**Acknowledgments**—We thank Dr. B. Vogelstein for generously providing CMV-p53, CMV-R175H, and 14-3-3$\alpha$ (BDS-2, 3X) promoter luciferase constructs and Dr. J. Brady for the GST-p53 expression construct. We also thank Dr. K. T. Jeang for providing CMV-Tat that was used in immunofluorescent microscopy experiments. Dr. Y. Nakatani for permission to use CMV-P/CAF, CMV-E1A 12S, and CMV-E1A 12S$\alpha$ expression constructs, and Dr. B. Nair for purified anti-Tat antibody, C-2145.

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