The Presence of HIV-1 Tat Protein Second Exon Delays Fas Protein-mediated Apoptosis in CD4\(^+\) T Lymphocytes

A POTENTIAL MECHANISM FOR PERSISTENT VIRAL PRODUCTION\(^*\)\(^\circ\)\(^\bullet\)

Received for publication, August 6, 2012, and in revised form, January 25, 2013. Published, JBC Papers in Press, January 30, 2013, DOI 10.1074/jbc.M112.408294

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Background: HIV-infected T cells are quite resistant to apoptosis.

Results: Intracellular expression of HIV-1 Tat in T cells stabilized the mitochondrial membrane and reduced caspase activation mainly through NF-\(\kappa\)B activation.

Conclusion: Intracellular Tat induced resistance to FasL-mediated apoptosis in T cells mainly through the second exon.

Significance: Tat-mediated protection against apoptosis may be a mechanism for HIV-1 persistence.

HIV-1 replication is efficiently controlled by the regulatory protein Tat (101 amino acids) and codified by two exons, although the first exon (1–72 amino acids) is sufficient for this process. Tat can be released to the extracellular medium, acting as a soluble pro-apoptotic factor in neighboring cells. However, HIV-1-infected CD4\(^+\) T lymphocytes show a higher resistance to apoptosis. We observed that the intracellular expression of Tat delayed FasL-mediated apoptosis in both peripheral blood lymphocytes and Jurkat cells, as it is an essential pathway to control T cell homeostasis during immune activation. Jurkat-Tat cells showed impairment in the activation of caspase-8, deficient release of mitochondrial cytochrome c, and delayed activation of both caspase-9 and -3. This protection was due to a profound deregulation of proteins that stabilized the mitochondrial membrane integrity, such as heat shock proteins, prohibitin, or nucleophosmin, as well as to the up-regulation of NF-\(\kappa\)B-dependent anti-apoptotic proteins, such as BCL2, c-FLIP\(_S_\), XIAP, and C-IAP2. These effects were observed in Jurkat expressing full-length Tat (Jurkat-Tat101) but not in Jurkat expressing the first exon of Tat (Jurkat-Tat72), proving that the second exon, and particularly the NF-\(\kappa\)B-related motif ESKKKVE, was necessary for Tat-mediated protection against FasL apoptosis. Accordingly, the protection exerted by Tat was independent of its function as a regulator of both viral transcription and elongation. Moreover, these data proved that HIV-1 could have developed strategies to delay FasL-mediated apoptosis in infected CD4\(^+\) T lymphocytes through the expression of Tat, thus favoring the persistent replication of HIV-1 in infected T cells.

The human immunodeficiency virus type 1 (HIV-1) infection is characterized by a continuous viral replication in CD4\(^+\) T lymphocytes and macrophages (1), leading ultimately to the development of the acquired immunodeficiency syndrome (AIDS). This is caused by a progressive depletion of CD4\(^+\) T lymphocytes through different mechanisms such as apoptosis, cellular syncytia, plasma membrane disruption by viral budding, or cytotoxicity by soluble viral proteins as Tat, Nef, and gp120 (2–4). Vpr may also have a cytotoxic effect on bystander or infected CD4\(^+\) T cells by increasing the mitochondrial membrane permeabilization (5–8), although it has also been described as an anti-apoptotic factor (9, 10). The programmed cell death or apoptosis of CD4\(^+\) T cells during HIV-1 infection is a complex process that affects differently both infected and uninfected cells. Apoptosis occurs mainly in bystander noninfected cells, whereas productively HIV-1-infected cells have evolved strategies to prevent or delay apoptosis in the context of immune activation (11–14).

HIV-1 regulator Tat induces apoptosis of bystander cells when is released to the extracellular medium as a soluble form (15). However, when Tat is expressed intracellularly, it produces the efficient elongation of the viral transcripts through the recruitment of the RNA polymerase II complex. Tat binds to a stem-loop RNA termed Tat-response element, located at the 5’ end of the nascent viral transcripts (16), and activates the recruitment of cellular elongation factors such as P-TEFb,
increasing the processivity of RNA polymerase II (17). Tat is a 101-residue-long protein codified by two exons as follows: the first exon codifies amino acids 1–72, forming the transcriptionally active protein Tat72; and the second exon codifies amino acids 73–101 and overlaps with the env gene (18, 19). The Tat first exon (1–72 amino acids) contains the minimal functional domain to generate a protein competent in HIV-1 replication through a Tat-response element-dependent activation of the transcription, and the second exon (73–101 amino acids) has been described as dispensable for Tat activity (20). However, expression of the Tat second exon is conserved in all lentivirus, suggesting biological importance. In fact, the second exon is essential for Tat-mediated cell genome deregulation, thereby indicating that it may control the transcription of nonviral genes (Tat-response element-independent activation) (21–23), probably through binding to canonical enhancer sequences of cellular transcription factors such as NF-κB or Sp1 (24–26). This would indirectly affect the expression of several genes related to cellular functions such as T cell activation or apoptosis (15, 23, 27, 28). Moreover, the important contribution of Tat second exon to HIV-1 in vivo replication was demonstrated by the accidental infection of three laboratory workers with the HIV-1 HXB2 isolate, which shows a premature stop codon at the residue 89 (29, 30). In one of the infected patients, the HXB2 virus with first exon Tat reverted to second exon Tat (30), changing the mild course of the infection to a steep decline in CD4⁺ T cell count and a rapid progression to AIDS within 1 year (29). This unfortunate situation provided conclusive evidence for the biological requirement of the Tat second exon for HIV-1 replication and pathogenesis in vivo.

The role of Tat in apoptosis of bystander and infected cells is controversial because, although soluble Tat has been described as an inducer of apoptosis (15, 31, 32), it has also been proved to be a protector against apoptosis when it is expressed inside the host cell (28, 33). Our group demonstrated previously that intracellular Tat profoundly deregulates cellular gene expression, modifying the expression of genes involved in apoptosis (23). This deregulation was mainly due to the presence of the second exon, proving that although the first exon is sufficient for activating viral replication, full-length Tat should exert further control on HIV-1 pathogenesis by protecting the host cells against apoptosis. Apoptosis is essential to control T cell homeostasis, especially during the contraction of the immune response (34). As the antigen wanes, the number of T cells is appropriately reduced through the induction of apoptosis by Fas (CD95 or Apo-1), a member of the tumor necrosis factor receptor superfamily (35). Engagement of Fas receptor/CD95 with Fas ligand (Fasl)/CD178 or Fas-activating antibodies (anti-CD95) recruits procaspase-8 to death-inducing signaling complex (DISC)⁴ through the FADD adapter protein. Within DISC, procaspase-8 is activated by dimerization and autocleavage (36–38). After that, Fas may activate apoptosis through two different pathways that distinguish type I and type II cells. Most cell types are classified as type I, where caspase-8 directly activates caspase-3, the main effector of the morphological and biochemical changes characteristic of apoptosis (36, 37). In type II cells, Fas receptors are excluded from lipid rafts and assemble DISC inefficiently upon activation of death receptors (39). Consequently, only a small fraction of procaspase-8 is activated; therefore, they require a subsequent amplification step through the mitochondrial cell death pathway. The mitochondria are highly involved in the induction of apoptosis in mammalian cells as they release to the cytosol many important factors that induce caspase activation and chromosome fragmentation after the permeabilization of the mitochondrial outer membrane (40, 41). This mechanism is controlled by profound anti-apoptotic members of the BCL2 family such as the “BH3-domain only” protein Bid (42). Caspase-8-mediated cleavage of Bid initiates the mitochondrial cell death pathway (43–45) as truncated Bid (tBid) translocates to the mitochondrial membrane and triggers the release of cytochrome c to the cytosol (46). Cytochrome c participates then in the assembly of the apoptosome, a multiprotein complex required for caspase-9 activation that subsequently activates caspase-3 (47, 48).

Apoptosis is tightly controlled by many cellular proteins at different levels, and the final cell death occurs by imbalance between pro- and anti-apoptotic factors. One central regulator of cell survival and apoptosis is the transcription factor NF-κB that regulates the expression of anti-apoptotic genes such as BCL2, c-FLAP, XIAP, as well as the cellular FLICE-inhibitory protein (c-FLIP) (49). Upstream in the apoptotic pathway, c-FLIP interferes with Fasl-mediated activation through the binding to FADD and/or caspase-8/10 in a ligand-dependent manner, preventing DISC formation (50). There are three functional c-FLIP splice variants as follows: short form (c-FLIPₛ, 26 kDa) (51); intermediate or Raji form (c-FLIPᵢᵣ, 43 kDa) (52); and long form (c-FLIPᵢ, 55 kDa) (53). c-FLIPₛ is exclusively a caspase-8 inhibitor, whereas c-FLIPᵢᵣ has dual function as caspase-8 inhibitor or activator, depending on the different ratios of c-FLIPᵢ/caspase-8 (54). The mechanisms by which intracellular Tat interferes with apoptosis are not well known, although it has been described that Tat may enhance the expression of anti-apoptotic factors such as c-FLIP (33) or BCL2 (55, 56). The role of the second exon in the ability of Tat to protect against apoptosis is completely unknown.

It was determined that the intracellular expression of full-length Tat was able to delay Fas-mediated apoptosis in both PBLs and Jurkat and that this effect was due to the presence of the second exon. The mechanism of protection was based on the following: first on the deregulation of several NF-κB-dependent proteins, including the overexpression of BCL2 and c-FLIPₛ, and second on the preservation of the mitochondrial outer membrane integrity by several anti-apoptotic factors, delaying the release of cytochrome c and subsequent activation of caspase-9 and caspase-3. Getting further insight on this mechanism of protection against apoptosis in CD4⁺ T cellsmediated by intracellular full-length Tat would provide a better understanding of the role of Tat in the ability of HIV-1 to create a persistent infection in the host.

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⁴The abbreviations used are: DISC, death-inducing signaling complex; PBL, peripheral blood lymphocyte; PE, phycoerythrin; PI, propidium iodide; ANOVA, analysis of variance; PARP, poly(ADP-ribose) polymerase; RLU, relative light unit; tBid, truncated Bid; PHB, probitin; EYFP, enhanced YFP.
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EXPERIMENTAL PROCEDURES

Cells—PBLs were isolated from the blood of healthy donors by centrifugation through a Ficoll-Hypaque gradient (GE Healthcare). Jurkat E6-1 cells were obtained from the AIDS Reagent Program, National Institutes of Health (57). Jurkat-Tat72 and Jurkat-Tat101 stably express, respectively, the HIV-1 Tat first exon (1–72 amino acids) or full-length Tat (1–101 amino acids) by using a Tet-Off system Clontech. Jurkat Tet-Off cells transfected with empty vector pTRE2hyg were used as negative control. Jurkat-Tat72 and Jurkat-Tat101 are not clones but are mixed populations in which more than 50% of the cells express high amounts of intracellular Tat101 or Tat72 protein. It was determined that the expression of Tat in Jurkat-Tat101 and Jurkat-Tat72 was very similar to a real infection performed in MT-2 cells infected with the NL4.3WT strain (23). Both PBLs and Jurkat were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin (BioWhittaker, Walkersville, MD). In Jurkat-Tat cells, the culture medium was supplemented with 300 μg/ml geneticin (Sigma) and 300 mg/ml hygromycin B (Clontech).

Reagents and Antibodies—A monoclonal antibody against HIV-1 Tat (amino acids 2–9) was obtained from Advanced Biotechnologies Inc. (Columbia, MD). A monoclonal antibody against human Fas receptor (MBL International, Woburn, MA; clone SY-001) was used at 50 or 500 ng/ml during 4 or 18 h at 37 °C for inducing cell death in Jurkat or PBLs, respectively, and for quantifying the amount of Fas receptor on the cell surface by flow cytometry. A polyclonal antibody against caspase 3 (p32) and active subunits p17 and p20 (clone H-277) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against caspase 8 (p55) (clone 90A992) was also obtained from Santa Cruz Biotechnology. A monoclonal antibody against caspase 9 (p46) and active p35/p37 (clone 5B4) was obtained from Abcam (Cambridge, UK). Antibodies against BCL2 (clone C-2), Bid (clone 5C9), poly(ADP-ribose) polymerase (PARP-1) (clone 194C1439), c-FlipS/L (clone G-11), and p65/RelA (clone C-20) were obtained from Santa Cruz Biotechnology. The monoclonal antibody against cytochrome c was obtained from Calbiochem. A monoclonal antibody against β-actin (clone AC-15) was obtained from Sigma. Propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) were also obtained from Sigma. MitoTracker Red CMxRos mitochondrial probe was obtained from Invitrogen (Eugene, OR). Secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from GE Healthcare. Secondary IgG/IgM antibody fluorescent-conjugated (FITC) used in flow cytometry assays was purchased from Jackson ImmunoResearch (West Grove, PA).

Transient Transfection and Vectors—Resting PBLs and Jurkat cells were transiently transfected with an Easyjet Plus Electroporator (EquiBio, Middlesex, UK). In brief, 20 × 10⁶ PBLs or 10⁷ Jurkat were collected in 350 μl of RPMI 1640 medium without supplement and mixed with 1 μg/10⁶ cells of plasmid DNA. Cells were transfected in a cuvette with a 4-mm electrode gap (EquiBio) at 340 V for PBLs and 280 V for Jurkat, 1500 microfarads, and maximum resistance.

pCMV-Tat72 tag or pCMV-Tat101 tag expression vectors were obtained by cloning Tat72 and Tat101, obtained from the pCMV-Tat101 vector (58), in pCMV tag (Stratagene, Agilent Technologies, Madrid, Spain), in NotI/BamHI pNL4.3-TatM1, which contains a point mutation in the start codon of the tat gene, was obtained from pNL4.3 wild-type (WT) vector (kindly provided by Dr. M. A. Martin (59)) by site-directed mutagenesis with the QuikChange site-directed mutagenesis kit (Stratagene), using the following oligonucleotide: 5′-TCGAGAGGAGGACAGAACATTGAGCCAGTAGCT-3′, which introduced a point mutation (underlined) in the start codon, changing a methionine by an isoleucine (supplemental Fig. 1). The presence of the selected mutation was confirmed by automatic sequencing.

pNL4.3-TatM1II vector was co-transfected along with pCMV-Tat72 or pCMV-Tat101 in a ratio of 2:1. pEYFP-C1 vector (Clontech) was co-transfected as control of transfection efficiency and measured by flow cytometry.

Quantitative PCR—Total RNA was isolated with RNeasy mini kit (Qiagen Iberia, Madrid, Spain), and cDNA was synthesized by using the GoScript Reverse Transcription System (Promega), according to manufacturer’s instructions. HIV-1 transcription was determined by quantitative PCR analysis of all viral mRNAs using the following primers directed against env/nef genes: P3 (5′-TTTGCTCAATGCCACAGCCCAT-3′) and P4 (5′-TTTGACCACTTGGCCACCCAT-3′) (60). The expression of β-ACTIN was used as housekeeping gene to calculate the relative expression of env/nef genes. Primers for amplifying the human β-ACTIN gene were β-actin sense (5′-AGGCCCCAGAAGAGAGGCA-3′) and β-actin antisense (5′-GCCAGTCATTGTAAGGTGTTGCT-3′). SYBR Green PCR master mix (Applied Biosystems) was used according to manufacturer’s instructions.

Analysis of Apoptosis—Cells were stained with 1.5 μM PI or annexin-V conjugated with fluorescein isothiocyanate (FITC) (Immunostep, Salamanca, Spain), and fluorescence was measured by a FACSCalibur flow cytometer (Clontech). Data were analyzed by CellQuest software. Cell viability was also determined by using the CellTiter-Glo® luminescent cell viability assay (Promega), following the manufacturer’s instructions. Briefly, 1 × 10⁵ cells were harvested by centrifugation, washed twice with 1 × PBS, and resuspended in lysis buffer. After incubation for 10 min at room temperature to stabilize the luminescent signal, cell lysates were deposited in an opaque-walled multiwell plate and analyzed in an Orion microplate luminometer with Simplicity software (Berthold Detection Systems, Oak Ridge, TN).

Immunoblotting Assays—Nuclear and cytosolic protein fractions were obtained as described previously (61). Protein extracts were fractionated by SDS-PAGE and transferred onto a Hybond-ECL nitrocellulose paper (GE Healthcare). After blocking and incubation with primary and secondary antibodies, proteins were detected by chemiluminescence with SuperSignal West Pico/Femto chemiluminescent substrate (Pierce). Densitometry was performed in a Gel Doc 2000 System (Bio-Rad) by using Quantity One software. Gel bands were quantified, and background noise was subtracted from the images.
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The relative ratio of the optical density units corresponding to each sample was calculated regarding the internal control (β-actin) per each lane.

**Immunoﬂuorescence Assays**—Living cells treated or not with anti-CD95 for 4 h were stained with MitoTracker and then adhered onto PolyPrep slides (Sigma) and ﬁxed with 2% paraformaldehyde in 1× PBS. Immunoﬂuorescence assays were then performed as described previously (23). Images were obtained with a Leica DMI 4000B Inverted Microscope (Leica Microsystems, Barcelona, Spain).

Measurement of Caspase Activity—Activity of caspase-3/-7, -8, and -9 was measured with CaspaseGlo®3/7, CaspaseGlo®8, and CaspaseGlo®9 systems (Promega Biotech Iberica, Madrid, Spain), respectively. The luminescent signal (relative light units (RLUs)), which was directly proportional to caspase activation, was measured in an Orion microplate luminometer with Simplicity software (Berthold Detection Systems), and data were normalized according to the protein concentration in each sample.

Mitochondrial Cytochrome c Depletion—The release of cytochrome c from the mitochondria intermembrane space to the cytosol was measured by using InnoCyte flow cytometric cytochrome c release kit (Calbiochem). Brieﬂy, 5 × 10^6 cells treated or not with FasL were permeabilized and then ﬁxed with paraformaldehyde. After staining with a primary monoclonal antibody against cytochrome c and a secondary antibody conjugated with FITC, the measurement of cytochrome c release was performed by ﬂow cytometry.

Mitochondrial Membrane Potential Gradient (∆Ψm) was measured by ﬂow cytometry using MitoProbe JC-1 assay kit (Molecular Probes), according to manufacturer’s instructions. Brieﬂy, 2 × 10^6 cells/ml were incubated in JC-1 staining solution, and both green and red ﬂuorescence were measured in FL1 and FL2 channels, respectively, by ﬂow cytometry. The mitochondrial depolarization was calculated by measuring the decrease in the red/green ﬂuorescence intensity ratio.

NF-κB Activation Assays—The ability of NF-κB to bind DNA was measured in nuclear protein extracts by DNA afﬁnity immunoblotting assay, as described previously (23, 62). The quantity of NF-κB bound to DNA was detected by immuno blotting with a polyclonal antibody against p65/RelA (Santa Cruz Biotechnology). As internal control, an input of nuclear protein for each sample was analyzed by using an antibody against β-actin (Sigma).

NF-κB transactivation activity was measured by transient transfection with p3κB-LUC vector, which contains a luciferase gene under the control of three κB consensus sites from the immunoglobulin κ-chain promoter, as described before (23, 58). After incubation for 18 h, cells were treated or not with 50 ng/ml anti-CD95 for 4 h, and then the luciferase activity was measured by using luciferase assay system (Promega) in a Sirius luminometer (Berthold Detection Systems), according to the manufacturer’s instructions. The RLUs were normalized with protein concentration in each sample and with the percentage of efﬁciently transfected cells by using pEYFP-C1 vector as transfection internal control.

*Tat101 Site-directed Mutagenesis*—Site-speciﬁc mutagenesis of the 85ESKKKVE91 sequence necessary for Tat-mediated NF-κB activity, located in Tat second exon, was performed in the pTRE2hyg-Tat101 vector (28) by using QuikChange site-directed mutagenesis kit (Stratagene, Agilent Technologies), according to manufacturer’s instructions. The following oligonucleotide was used to introduce selected mutations (underlined) in the 85ESKKKVE91 motif to generate the motif 85ESRVNVY91: 5’-AGACCGAATCGAGGTGAACTGGTGAGAGAGACAGAGACAGAT-3’. The presence of the selected mutations was conﬁrmed by automatic sequencing.

**Proteome Profiling**—Two hundred micrograms of protein extracts were subjected to reduction and alkylation and then digested with trypsin (Promega) in 50 mM ammonium bicarbonate, pH 8.8. The resulting tryptic peptides were loaded into the liquid chromatography/tandem mass spectrometry (LC-MS/MS) system and analyzed by using a C-18 reversed phase nano-column (75-mm inner diameter × 25 cm, 3-mm particle size, Acclaim PepMap 100 C18, Thermo Fisher) in a continuous acetonitrile gradient consisting of 0–30% B in 145 min, 30–43% A in 5 min, and 43–90% B in 1 min (A = 0.5% formic acid; B = 95% acetonitrile, 0.5% formic acid). A flow rate of ~300 nl/min was used to elute peptides from the reverse phase nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on an orbital ion trap mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientiﬁc) (63). An enhanced resolution spectrum (resolution = 60,000) followed by the MS/MS spectra from the ﬁve most intense parent ions were analyzed during the chromatographic run (180 min). Dynamic exclusion was set at 0.5 min. For peptide identiﬁcation, all spectra were analyzed with Proteome Discoverer (version 1.2, Thermo Fisher Scientiﬁc), using both Sequest (Thermo Fisher Scientiﬁc; version 1.0.43.2) (64) and Mascot search engines. For database searching, a Uniprot database was interrogated selecting the following parameters: trypsin digestion with two maximum missed cleavage sites, precursor mass tolerance of 20 ppm, fragment mass tolerance of 1200 millimass units, carbamidomethylcysteine as ﬁxed modiﬁcation, and methionine oxidation, asparagine deamidation, and serine and threonine phosphorylation as dynamic modiﬁcations. For peptide and protein identiﬁcation validation, results were loaded into Scaffold 3.0 software (Proteome Software Inc., Portland, OR), adding X!Tandem as an additional search engine. Sequest search engine facilitates the “XCorr score” (values above 2.0), which measures how close the spectrum detected in the sample ﬁts to the ideal spectrum. Differential expression in samples was determined using the quantity values of normalized spectra for each peptide, as determined by Scaffold software in a triplicate experiment. Peptide identiﬁcations were accepted if they could be established at greater than 95.0% probability as described by the Peptide Prophet algorithm (65). Only those proteins involved in apoptotic pathways, cell survival, and mitochondrial function were selected for further analysis.

**Antibody-based Apoptosis Microarray**—RayBio human apoptosis antibody array kit (RayBiotech Inc., Norcross, GA) detects the relative level of 43 apoptosis-related proteins in cell lysates by using an array of antibodies spotted on a glass chip. Procedure was performed according to manufacturer’s instructions. All microarrays were scanned under the same conditions in a ScanArray Express HT microarray scanner (PerkinElmer...
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Life Sciences) using Alexa 555-specific filters. Images were quantified by QuantArray analysis software and a fixed circle segmentation algorithm. Data were filtered to discard those data points that were not considered positive with respect to the local background and the local negative controls. A second filtering round was done by removing from further analyses those proteins with less than three passing data points. Data were normalized against the positive controls of each array and then were mean-aggregated to result in a single value for each analyzed protein. Normalized data points where then logarithmically transformed for data compression and a better visualization of the results. Finally, logarithmic ratios (log2 ratios) against the reference data obtained from control cells were generated. Only proteins that were deregulated in Jurkat-Tat101 and Jurkat-Tat72 versus Jurkat Tet-Off cells were represented.

Apopotosis-related Protein Networks—All proteins related to apoptosis that were deregulated were subjected to analysis with STRING 9.0 (66). Deregulated proteins were integrated in interconnected networks built by inputting the list of protein symbols, selecting Homo sapiens as organism and medium confidence, which does not consider interactions with combined scored under 0.4.

Statistical Analysis—Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Corporations between control and FasL treatment groups were made using two-way analysis of variance (ANOVA) with Bonferroni post-test analysis to describe the statistical differences among groups. p values <0.05 were considered statistically significant in all comparisons and were represented as *, **, or *** for p < 0.05, p < 0.01, or p < 0.001, respectively.

RESULTS

Apopotosis Induced by FasL Is Delayed in PBLs Transiently Transfected with Tat101 Alone or in the Context of HIV-1 Genome—PBLs from healthy donors were transfected with pCMV-Tat101 or pCMV-Tat72 expression vectors. Transfection with pcDNA3 was used as negative control, and pEYFP-C1 was co-transfected in all cases as a control of transfection efficiency. Viability after transfection was 40–45% (data not shown), and transfection efficiency was 20–25%, depending on the batch of PBLs. Transfected PBLs were cultured for 3 days and then treated with FasL for 18 h. Viability was measured by chemiluminescence. Media from three different independent experiments are represented in Fig. 1A. It was observed that the intracellular expression of Tat101 in PBLs was able to exert 2.0-fold more protection against FasL-mediated apoptosis than control cells (p < 0.01), whereas Tat72 exerted lower protection. The efficient expression of Tat72 and Tat101 was assessed in EYFP⁺ cells by immunofluorescence (Fig. 1B), using DAPI to stain the nucleus, where most Tat was localized. To evaluate whether the resistance to apoptosis occurred mainly on CD4⁺ T cells, the transfected PBLs challenged with FasL for 18 h were stained with an antibody against CD4 conjugated with phycoerythrin (PE) and then analyzed by flow cytometry. PI⁻ cells were gated and EYFP⁺/CD4⁺ cells were quantified within this group. It was determined that Tat101 alone was able to induce 2-fold more protection against FasL-mediated apoptosis than Tat72 or empty vector pcDNA3 in CD4⁺ T cells (Fig. 1C).

![FIGURE 1. FasL-mediated apoptosis was reduced in PBLs expressing intracellular Tat101. A, resting PBLs were transiently transfected with pCMV-Tat72 or pCMV-Tat101 expression vectors or with pcDNA3 as negative control. pEYFP-C1 was used as control of transfection efficiency. PBLs were maintained for 3 days without stimulus, and then were treated with FasL for 18 h. Viability was measured by chemiluminescence. The bar diagram represents the media of three independent experiments, and lines on top of the bars correspond to the mean ± S.E. Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis; ** indicates p < 0.01. B, Tat expression and nuclear localization were confirmed by immunofluorescence. DAPI was used for nuclear staining. C, resting PBLs were transiently transfected with pCMV-Tat72 or pCMV-Tat101 expression vectors or with pcDNA3 as negative control. pEYFP-C1 was used as control of transfection efficiency. PBLs were maintained for 3 days without stimulus and then were treated with FasL for 18 h. Cells were stained with a monoclonal antibody against CD4 conjugated with PE and then with PI. Signals corresponding to EYFP, PE, and PI were analyzed by flow cytometry in FL1, FL2, and FL3 channels, respectively. SSC/FSC dot plot was used to select living (PI⁻) cells (region R1) as follows: red events correspond to PI⁺ living cells; magenta events correspond to PI⁺ dead cells; black events correspond to cellular debris. The numbers in the PI⁻/EYFP⁺/CD4⁺ dot plots show the percentage of CD4⁺/EYFP⁺ cells within the PI⁻ region (region R3). The bar diagram represents the media of three independent experiments, and the lines on top of the bars correspond to the mean ± S.D.](image-url)
transfected as control of transfection efficiency, which was 20–25% in all cases. HIV-1 replication was assessed by quantitative RT-PCR. Efficient expression of Tat72 and Tat101 was assessed by immunofluorescence (data not shown). Transfected Jurkat cells were cultured for 3 days and then treated with FasL for 4 h. Cell viability was measured by luminometric assay, and the media of three different independent experiments were represented as a ratio of the apoptosis induced in FasL-treated PBLs regarding untreated cells (Fig. 3A, 1st bar diagram). It was observed that the intracellular expression of Tat101 in Jurkat cells produced 1.8-fold more protection against FasL-mediated apoptosis than control cells, whereas Tat72 exerted lower protection ($p < 0.05$).

To dissect the mechanism underlying intracellular Tat-mediated protection against apoptosis by FasL in T cells, the use of Jurkat-Tat101 and Jurkat-Tat72 stable cell lines was analyzed. Jurkat-Tat101 and Jurkat-Tat72 were treated with FasL at different times, and early apoptosis was measured by annexin-V-FITC staining (Fig. 3B). Flow cytometry analysis showed that the percentage of pro-apoptotic cells was 1.8-fold lower on average in Jurkat Tat101 than in control cells, whereas this percentage in Jurkat Tat72 was 1.2-fold lower on average than control.

Measurement of apoptosis by PI staining in Jurkat-Tat72 and Jurkat-Tat101 treated with FasL for 4 h showed that Tat101 delayed 4.6-fold the induction apoptosis, whereas Tat72 delayed apoptosis 1.7-fold (Fig. 3C). The expression of Fas receptors/CD95 on the cell surface was increased by 20% in Tat101, but Tat 72 did not produce significant changes (Fig. 3D).

**Fas-mediated Activation of Caspase-8 and -3 Is Decreased in Jurkat-Tat101 Cells**—Cleavage of procaspase-3 was analyzed by immunoblotting in protein extracts obtained from Jurkat-Tat101, Jurkat-Tat72, and control cells treated with FasL for 4 or 18 h. As shown Fig. 4A, the active subunits p17 and p11 were rapidly generated in Jurkat-Tat72 and control cells after treatment with FasL for 4 h. After 18 h of treatment, the cleavage of caspase-3 was also initiated in Jurkat-Tat101, although 2-fold more of high quantity unprocessed precursor p32 could also be observed in these cells.

To further evaluate the activation of caspase-3 in Jurkat-Tat cells treated with FasL, the cleavage of the caspase-3 substrate PARP-1 was analyzed by immunoblotting. PARP-1 cleaved from p89 was abundantly detected in the cytoplasm of control cells treated with FasL for 4 h, but it was only weakly produced in Jurkat-Tat101 even after treatment for 18 h (Fig. 4B). In Jurkat-Tat72, the levels of cytosolic p89 were halfway between those detected in Jurkat-Tat101 and control cells. Measurement of caspase-3/-7 activity showed that the activation of those caspases in response to FasL was delayed in Jurkat-Tat101 regarding Jurkat-Tat72 and control cells ($p < 0.05$) (Fig. 4C).

**Caspase-3/-7 Activity** showed that the activation of these caspases in response to FasL was delayed in Jurkat-Tat101 regarding Jurkat-Tat72 and control cells ($p < 0.05$) (Fig. 4C). Measurement of the activation of caspase-8 showed 1.6-fold reduction in Jurkat-Tat101 ($p < 0.001$), whereas the difference between Jurkat-Tat72 and control cells was lower (Fig. 4D). The cleavage of caspase-8 by immunoblotting showed in Jurkat-Tat101 a great delay in the cleavage of procaspase-8 (p55) after treatment with FasL for 18 h. This was not observed in Jurkat-Tat72.
Release of Cytochrome c and Activation of Caspase-9 Are Impaired in Jurkat-Tat101 Cells Due to High Stability of the Mitochondrial Inner Membrane Electrochemical Potential—Analysis by flow cytometry showed that the release of cytochrome c to the cytoplasm from the mitochondrial intermembrane space in response to treatment with FasL for 4 or 18 h was reduced nearly 2-fold in Jurkat-Tat101 cells, whereas no significant difference with control cells was observed in Jurkat-Tat72 cells (Fig. 5A). These results were confirmed by confocal microscopy through the analysis of the subcellular localization of cytochrome c (Fig. 5B). Consistent with data obtained by flow cytometry, the mitochondria of Jurkat cells with stable expression of Tat101 were more resilient to release cytochrome c in response to FasL. Besides, the mitochondria of Jurkat-Tat101 cells showed a more diffuse and less compact distribution than those in Jurkat-Tat72 and control cells.

Cytosolic protein extracts for Jurkat-Tat101, Jurkat-Tat72, and control cells treated with FasL for 4 or 18 h were analyzed by immunoblotting using an antibody against precursor p46 and active forms p37/p35 of caspase-9. As shown Fig. 6A, p37/p35 were only detected in control cells and weakly in Jurkat-Tat72, and control cells were treated with FasL during 4 h and then stained with PI. The induction of apoptosis was measured by flow cytometry. Region R1 was used to select the living PI− cells in the groups of untreated cells, displayed as SSC/FSC dot plots. The percentage of apoptotic cells (PI+) was determined within R1 in treated cells and then represented as histograms where the basal cell death (continuous line) was compared with the apoptosis induced after treatment with FasL (discontinuous line). The numbers shown correspond to the percentage of cells expressing CD95 after subtracting the isotype control. All data shown are media or representative of three independent experiments.
confirmed by measuring the activity of caspase-9 by chemiluminescence, which was 1.7-fold lower in Jurkat-Tat101 cells ($p < 0.001$) (Fig. 6B).

The mitochondrial membrane potential gradient ($\Delta \Psi_{m}$) was measured by using the cationic lipophilic dye JC-1 (68). The dispersion of green JC-1 monomers from the mitochondria throughout the entire cell, which correlates with the induction of apoptosis, was measured by flow cytometry after treatment with FasL for 4 or 18 h (Fig. 6C). The population of Jurkat-Tat101 cells committed to apoptosis, low $\Delta \Psi_{m}$ and higher monomeric JC-1 with green fluorescence, was smaller than in Jurkat-Tat72 and control cells after treatment with FasL. This difference was mainly observed after 18 h of treatment, where nearly 30% of Jurkat-Tat101 cell population showed high resistance to lose the mitochondrial membrane potential, regarding control cells. In accordance, the population of living cells with higher $\Delta \Psi_{m}$, where JC-1 formed aggregates with red fluorescence, was also 2.8-fold greater in Jurkat-Tat101.Jurkat-Tat72 cells showed an intermediate profile of mitochondrial membrane integrity loss with 1.8-fold higher $\Delta \Psi_{m}$ than control cells. As a result, the mitochondrial depolarization, measured by the decrease in the red/green fluorescence intensity ratio, was delayed by the intracellular expression of Tat101 and, with less intensity, by Tat72.

Analysis by immunoblotting of BCL2 showed that the expression was enhanced in Jurkat-Tat101 in basal conditions, and its quantity was not significantly reduced even after treatment with FasL during 18 h (Fig. 6D). The expression of Bid (p22) diminishes as caspase-8 cleaves it to generate the truncated form tBid/p15 that translocates to the mitochondria and directly induces the outer membrane permeabilization (43). The expression of Bid/p22 was significantly reduced in Jurkat-Tat72 and control cells after treatment with FasL, suggesting that tBid was being synthesized and translocated to the mito-
chondrial membrane. However, Bid/p22 remained uncleaved in Jurkat-Tat101, proving that the activation of caspase-8 was deficient in these cells.

**Tat Deregulates the Expression of Cellular Proteins Related to Apoptosis and to the Maintenance of Mitochondrial Outer Membrane Integrity**—The whole proteome of Jurkat-Tat101, Jurkat-Tat72, and control cells was analyzed by LC-MS/MS in basal conditions. The analysis showed that Tat mostly induced the up-regulation of proteins related to the cell cycle and proliferation such as PHB, HEBP2/SOUL, NUDC, MCM2/3, PCNA, and NASP (Table 1). The expression of most of these proteins was more enhanced by the expression of Tat101 than Tat72. Besides, proteins related to apoptosis and stress response were also up-regulated by Tat, such as 14-3-3/H9256/H9254, Bid, DJ-1/PARK7, and several heat shock proteins. More than 50% of the apoptotic proteins deregulated were related to the mitochondrial function, including ATP synthase α and β subunits, which were mainly enhanced by the expression of Tat101. Most of these proteins belong to interconnected cellular pathways, and in some cases their activation was related to caspase-3 activity. This was observed after analyzing the predicted protein interactions by STRING 9.0 database. As shown Fig. 7, caspase-3 was directly connected to Bid, HSPE1, HSPD1, YWHAZ, and PCNA. In turn, these proteins were connected with the other proteins whose expression was modulated by Tat, forming an intertwined network that included mostly all deregulated proteins.

**Intracellular Tat101 Deregulates Several NF-κB-dependent Proteins Involved in the Control of Apoptosis**—The expression of c-FLIP<sub>L</sub>/S was analyzed by immunoblotting (Fig. 8A). c-FLIP<sub>L</sub>, rather than c-FLIP<sub>S</sub>, which is the one that confers resistance to human T cells against Fas-mediated apoptosis (69), was 2.1- and 2.8-fold enhanced in Jurkat-Tat72 and Jurkat-Tat101 in basal conditions. Treatment with FasL for 18 h reduced the expression of c-FLIP<sub>S</sub> similarly in all cell types, but the expression of c-FLIP<sub>L</sub> was very different depending on the expression of Tat or the treatment with FasL. Jurkat-Tat101 and Jurkat-Tat72 showed 10.5- and 3.8-fold more c-FLIP<sub>S</sub> expression than control cells, respectively, after treatment with FasL for 18 h. However, the expression of c-FLIP<sub>R</sub>/p43 was enhanced 22.5- and 12.5-fold more in Jurkat-Tat101 and Jurkat-Tat72, respectively, than in control cells, in basal conditions. After treatment with FasL, the cleavage of c-FLIP<sub>R</sub> to c-FLIP<sub>R</sub>/p43 was increased 30-fold more in both Jurkat-Tat101 and Jurkat-Tat72 (see supplemental Fig. 2).

As the expression of BCL2 and c-FLIP depends on NF-κB activity, the ability of the main NF-κB subunit p65/RelA to bind DNA was analyzed by DNA affinity immunoblotting assay in Jurkat-Tat101 and Jurkat-Tat72 cells in basal conditions, regarding control cells. As described previously, NF-κB binding
capacity was higher in Jurkat-Tat101 than in Jurkat-Tat72 and, in turn, higher in Jurkat-Tat72 than in control cells (Fig. 8B) (23, 70). This was reflected in the enhancement of the expression of T cell activation markers such as CD69, which was 3.3-fold increased in Jurkat-Tat101 ($p < 0.01$) and 1.5-fold in Jurkat-Tat72 cells, regarding control cells (Fig. 8C). Treatment with Fasl did not reduce significantly the expression of CD69 on the cell surface ($p < 0.05$). NF-κB transcriptional activity was also

FIGURE 6. Jurkat-Tat101 showed a reduced cleavage of Bid and procaspase-9, overexpression of BCL2, and higher stability of the mitochondrial inner membrane potential. A, cleavage of procaspase-9 was analyzed by immunoblotting in cytosolic protein extracts obtained from Jurkat-Tat101, Jurkat-Tat72, and control cells treated or not with Fasl during 4 or 18 h, using an antibody against the procaspase-9 (p46) and active caspase-9 (p37/p35) fragments. β-Actin was used as loading control. Gel bands were quantified by densitometry, and the background noise was subtracted from the images. The relative ratio of the optical density units corresponding to each sample was calculated regarding the internal control (β-actin) per each lane. The bar diagram shows the media of relative RLUs fold from three independent experiments, and the lines on the top of the bars represent the S.D. Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis, and *, **, and *** correspond to $p < 0.05$, $p < 0.01$, or $p < 0.001$, respectively. B, caspase-9 activity was measured by chemiluminescence. C, mitochondrial membrane potential gradient ($\Delta \Psi_{m}$) was measured by flow cytometry using the lipophilic dye JC-1. Red fluorescent aggregates in the undamaged mitochondria were represented in a dot plot versus the green fluorescent monomers dispersed through the cytosol. The numbers upper left in the diagrams represent the cells with stable mitochondrial membrane integrity; the numbers lower right represent the loss of mitochondrial membrane integrity. A representative experiment out of three is shown. D, expression of BCL2 and Bid (p22) was analyzed by immunoblotting, and β-actin was used as loading control.
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TABLE 1
Selected proteins differently expressed and detected by mass spectrometry in protein extracts from Jurkat-Tat101 and Jurkat-Tat72 cells, in comparison with Tet-Off cells

| Accession code | Name | Quantitative value | JJ Tat72 vs JJ TetOff | JJ Tat101 vs JJ TetOff | Biological process | Subcellular localization | Peptide Sequence & PTM | Protein probability | X-Curr score |
|----------------|------|--------------------|-----------------------|-----------------------|--------------------|-------------------------|------------------------|---------------------|-------------|
| gi|7661958 BCL2-associated transcription factor 1 (BCL-AF1) | 1.25 | 1.48 | Pro-apoptosis | Cytoplasm | Nucleus | LKDJDFY3QPLRM | KAEQGQPEEPQFGDKSSL (p) | 100% | 6.45 | 3.17 |
| gi|53458910 ATP synthase subunit beta (ATP5B) | 1.50 | 2.00 | ATF synthase | Mitochondrion | TIAAAGDQTEGLVR | VVDLALPAVK (p) | 99.8% | 1.7 | 1.32 |
| gi|5636988 ATP synthase subunit alpha (ATP5A1) | 1.25 | 2.00 | ATP synthase | Mitochondrion | ILGDAFTSVELETRVGLSGDGR | ILGDAFTSVELETRVGLSGDGR (p) | 100% | 4.43 | 2.73 |
| gi|3280420 Pyruvate kinase isoenzymes M1/M2 (PKM2) | -2.44 | 2.07 | Glycolysis | Cytoplasm | NADHPIGREL | NADHPIGREL (p) | 99.8% | 3.04 | 2.31 |
| gi|5557361 BH3 interacting domain death agonist (BID) | 1.00 | 2.43 | Pro-apoptosis | Cytoplasm | ELDAKICWLPVLAPVQGTYDGTR | EADNSQEDQR (p) | 99.8% | 6.48 | 2.73 |
| gi|5005623 106kDa heat shock protein (HSP10) | 2.61 | 2.45 | Stress response | Mitochondrion | DGDLELQVD | ELPEFR (p) | 100% | 1.50 | 1.50 |
| gi|234688 Heat shock protein 75 kDa (HSPA1A) | 2.22 | 2.63 | Stress response | Mitochondrion | DPDVVPATD | VQDLPQDEGR (p) | 99.8% | 3.28 | 2.47 |
| gi|2726268 Nuclear autoantigenic sperm protein (NASP) | 2.92 | 3.06 | DNA replication | Cell cycle | SYVEQGASELQGDRF | GVNLTVLQSDGDEAEAGGKEN | 99.8% | 6.48 | 2.64 |
| gi|7650593 14-3-3 zeta/delta (YWHAZ) Protein kinase C inhibitor 1 (PKCI-1) | 5.71 | 3.95 | Anti-apoptosis | Cytoplasm | IQEHEIQSVLYDSTSEYFKLNR I nuisanceQSVLYDSTSEYFKLNR | 99.8% | 6.48 | 2.64 |
| gi|3356547 DNA replication licensing factor MCM2 (MCM2) | 2.59 | 4.07 | DNA replication | Cell cycle | RGNIPTSGPR | RGNIPTSGPR | 99.8% | 3.18 | 2.47 |
| gi|3154294 DNA replication licensing factor MCM2 (MCM2) | 6.01 | 5.48 | Host-virus interaction | Mitochondrion | IQEHEIQSVLYDSTSEYFKLNR I nuisanceQSVLYDSTSEYFKLNR | 99.8% | 3.18 | 2.47 |
| gi|4056614 Progerin (PGN) | 16.10 | 20.50 | DNA replication | Cell proliferation | FSAGLGIQMGSR | YY1LPKKEDEEQG | 99.8% | 2.87 | 1.34 |
| gi|6631975 DNA replication licensing factor MCM2 (MCM2) | 1.00 | 26.80 | DNA replication | Cell cycle | DQKSYDVPQDKSTDEEMVYEPPK | 99.7% | 2.77 | 4.87 |
| gi|7650593 Nuclear migration protein nuC (NUDC) | 42.10 | 27.30 | Mitosis | Cell cycle | LSDLISER | LYSDELPENK | 99.8% | 2.18 | 3.1 |
| gi|7657603 Heme-binding protein 2 (HEPB2/SOUL) | 10.80 | 27.60 | Cell proliferation | Cytoplasm | AIEGTFVVR | SDFDPSAQK (p) | 99.8% | 3.15 | 2.12 |
| gi|565773 Prohibitin (PHB) | 49.40 | 71.70 | DNA synthesis | Cell cycle | NITYPAGQGSLQDLPQ | 99.8% | 7.96 | 3.61 |

Amino acid residues with post-translational modifications (PTMs) are underlined and in lowercase. PTMs detected were as follows: p, phosphorylation; a, acetylation; d, deamidation; and o, oxidation.
Proteins 70 kDa (HSPA1A/1B) and 60 kDa (HSPD1) were also upregulated in both Jurkat-Tat101 and Jurkat-Tat72 cells. Surprisingly, Jurkat-Tat101 also showed great quantities of pro-apoptotic factors such as lymphotoxin α or Bax, which were not detected in Jurkat-Tat72. On the contrary, the expression of BCL2 interacting mediator of cell death (BIM/BCL2L11) was diminished 2-fold in Jurkat-Tat101. The analysis of the predicted interactions between these proteins by STRING 9.0 database showed a connection between all of them and that p65/RelA had a central position in the center of this complex network (Fig. 8).

**DISCUSSION**

Activation of death signals is part of the antiviral immune response in the infected cell and represents a major threat to virus (71, 72). Many viruses have evolved mechanisms to inhibit apoptosis to the extent of the survival of infected cells, enhance the production of viral progeny, and permit the establishment of viral persistence. DNA viruses, such as adenovirus, Epstein-Barr, or African swine fever virus, encode viral anti-apoptotic proteins similar to cellular proteins, such as D-type cyclin, BCL2, and c-FLIP (73), whereas the herpes simplex virus type 1 (HSV-1) maintains the cellular BCL2 levels through different mechanisms, including the activation of p38 MAPK (74). It is not well known whether the RNA viruses have similar mechanisms, but HIV-1-infected cells appear to have developed certain resistance to apoptosis to prolong the viral production (13, 14). The molecular mechanisms underlying this protection are not fully comprehended, but the intracellular expression of viral regulatory proteins such as Vpr or Tat during the first step of the viral replication may be involved. Interestingly, both proteins seem to overlap their function, suggesting that preventing apoptosis during the first steps of the HIV-1 replication is a crucial step for the viral cycle and its pathogenesis. Vpr exerts a dual role on regulating apoptosis during HIV-1 infection because, although extracellular or high intracellular levels of Vpr induce cellular death through the alteration of mitocho-
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A

|          | J JTetOff | J JTat72 | J JTat101 |
|----------|-----------|----------|-----------|
| FasL     | 0         | 4        | 18        |
| c-FLIP<sub>L</sub> (p55) | *         |          |           |
| c-FLIP<sub>R</sub> (p43) |          | *        |           |
| c-FLIP<sub>S</sub> (p28) |           |          |           |
| β-actin  | 1.0       | 1.0      | 0.8       |
| Relative ratio | 1.0  | 1.0  | 2.2 |

B

|          | J JTetOff | J JTat72 | J JTat101 |
|----------|-----------|----------|-----------|
| p65/RelA | 1.0       | 1.5      | 2.2       |
| β-actin  | 1.0       | 1.0      | 1.0       |

C

|         | Control | J JTat72 | J JTat101 |
|---------|---------|----------|-----------|
| % CD69  | 0       | 40       | 70        |
| Untreated |        |          |           |
| FasL treated |    |          |           |

D

|         | Control | J JTat72 | J JTat101 |
|---------|---------|----------|-----------|
| Fold RLUs | 0       | 5        | 20        |
| Untreated |        |          |           |
| FasL treated (4h) |    |          |           |

E

F

|         | Control | Tat101 | Tat101 |
|---------|---------|--------|--------|
| Fold NF-κB activity |        |        |        |
| ESKKKVE | ESRVNVV |        |        |

|         | Control | Tat101 | Tat101 |
|---------|---------|--------|--------|
| % Apoptosis |        |        |        |
| ESKKKVE | ESRVNVV |        |        |
TABLE 2
Proteins differentially expressed and detected by human apoptosis antibody array in protein extracts from Jurkat-Tat72 and Jurkat-Tat101 in comparison with Tet-Off cells

| Protein Name                                                | Ratio JJ Tat72 versus JJ Tet-Off | Ratio JJ Tat101 versus JJ Tet-Off | Biological process                  | Subcellular localization       |
|-------------------------------------------------------------|----------------------------------|----------------------------------|-------------------------------------|-------------------------------|
| Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) | -10.00                          | -3.33                            | Pro-apoptosis                       | Cell membrane Secreted        |
| BCL2-interacting mediator of cell death (BIM)/BCL2L11         | 1.00                             | -2.70                            | NF-kB positive regulation           | Mitochondrion                 |
| Cyclin-dependent kinase inhibitor 1 (CDKN1A)/p21              | -1.75                            | -2.63                            | Cell cycle                          | Cytoplasm Nucleus             |
| Heat shock 70-kDa protein 1A/1B (HSPA1A/B)                    | 2.03                             | 1.50                             | Stress response                     | Cytoplasm Nucleus             |
| Tumor necrosis factor receptor superfamily member 6 (Fas)    | 2.05                             | 2.14                             | Anti-apoptosis                      | Cell membrane Secreted        |
| Baculoviral IAP repeat-containing protein 4 (XIAP)            | 2.14                             | 2.25                             | Anti-apoptosis                      | Cytoplasm Nucleus             |
| Insulin-like growth factor-binding protein 2 (IGFBP2)         | -2.2                             | 1.6                              | Cell growth regulation              | Secreted                      |
| Baculoviral IAP repeat-containing protein 3 (C-IAP2)          | 1.00                             | 39.12                            | Anti-apoptosis                      | Cell membrane Secreted        |
| Lymphotixin-α (LTA)/TNF-β (TNFB)                             | 1.00                             | 60.35                            | Pro-apoptosis                       | Secreted                      |
| Apoptosis regulator BAX/BCL2L4                                | 1.00                             | 101.21                           | Pro-apoptosis                       | Cytoplasm Mitochondrion       |
| 60-kDa heat shock protein (HSPD1)                             | 538.15                           | 462.85                           | Host-virus interaction              | Mitochondrion                 |

FIGURE 8. NF-kB-dependent proteins involved in the control of apoptosis were up-regulated in Jurkat-Tat101. A, expression of c-FLIP, (55 kDa), c-FLIP, (43 kDa), and c-FLIP, (28 kDa) isoforms was analyzed by immunoblotting in cytosolic protein extracts from Jurkat-Tat101, Jurkat-Tat72, and control cells treated or not with FasL for 4 or 18 h. β-Actin was used as loading control. B, NF-kB activity was analyzed in basal conditions by using DNA affinity immunoblotting assay. Gel bands were quantified by densitometry, and the background noise was subtracted from the images. The relative ratio of the optical density units corresponding to each sample was calculated according to the internal control (β-actin) per each lane. C, Jurkat-Tat72, Jurkat-Tat101, and control cells were treated with FasL during 4 h and then stained with an antibody against CD69 conjugated with PE. The percentage of cells expressing CD69 on the cell surface was measured by flow cytometry. D, NF-kB-dependent transactivation activity was measured by transient transfection of vector p3xLUC. Fold mean of RLUs corresponding to three independent experiments is represented, and lines on the top of the bars correspond to S.D. E, network of proteins related to apoptosis and identified by antibody-based microarray (see Table 2), expression of which was modified in Jurkat-Tat101 and Jurkat-Tat72 versus control cells. Predicted protein-protein interaction was obtained using a medium confidence score level (0.400). Data supporting protein-protein interactions derived from experimental studies (reddish purple lines), homology (light purple lines), databases (light blue lines), text mining (light green lines), and co-expression (black lines). The color of the nodes is arbitrary. F, Jurkat Tet-Off cells were transiently co-transfected with p3xLUC vector and pTRE2hyg-Tat101 E5KKR (wild type), pTRE2hyg-Tat101 ESRNVV (mutated), or pTRE2hyg empty vector as negative control. After 18 h, NF-kB activity was measured by chemiluminescence. Fold of relative RLUs regarding the control cells are represented (upper bar diagram). Cells were then treated with FasL for 4 h, and apoptosis was measured by PI staining and analyzed by flow cytometry (lower bar diagram). The bar diagrams show the media obtained from three independent experiments, and lines on the top of the bars represent the S.D. Two-way ANOVA with Bonferroni post-test analysis was performed for statistical analysis. Symbols *, **, and *** indicate p < 0.05, p < 0.01, and p < 0.001, respectively.
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caspase-8 and a lower cleavage of Bid but also due to the over-expression of other proteins such as BCL2 that may counteract this mechanism and stabilize the mitochondrial membrane (80, 81). BCL2 was described to be overexpressed by full-length Tat (55, 56), inducing protection against mitochondrionally mediated apoptosis in Jurkat (82). BCL2 expression did not decrease in Jurkat-Tat101 even after a long treatment with FasL. As the cleavage of Bid and BCL2 levels was quite similar in Jurkat-Tat72 than in control cells, this would explain the higher mitochondrial depolarization observed in these cells, reinforcing the assumption that the ability of Tat to protect against apoptosis mostly resided in the second exon.

However, BCL2 was not the only protein responsible for delaying apoptosis as other proteins overexpressed by Tat101 and Tat72 were also able to stabilize the mitochondrial membrane potential such as several mitochondrial heat shock proteins (HSPD1, HSPE1, and HSPA1), nucleophosmin (NPM1), and BCL2-associated transcription factor 1 (BCLAF1). Over-expression of HSPs has been related to an increase in BCL2, mitochondrial membrane potential stabilization, impairment of cytochrome c release, caspase-3 inhibition, and suppression of PARP cleavage (83–85). HSPE1 was acetylated at Lys-99 in Jurkat-Tat101, which has been related to a higher gene expression regulation (86). BCLAF1 was also overexpressed by Tat101 and Tat72 and was heavily phosphorylated at Ser-177 and Ser-512 mainly in Jurkat-Tat101. Recent studies proved that BCLAF1 has a major role in T cell activation (87, 88). Besides, nucleolar NPM1 suppresses apoptosis by inhibiting caspase-mediated activation of DNase, favoring genomic stability and DNA repair (89), as well as by blocking p53 mitochondrial localization (90). NPM1 is known to interact with HIV-1 Rev (91) and Tat, being critical for Tat nuclear localization and Tat-mediated transcription (92). Although NPM1 was overexpressed in both Jurkat-Tat101 and Jurkat-Tat72, it was deamidated at Asn-210 in Jurkat-Tat72; therefore, it was not fully functional in these cells. NPM1 also enhances the expression of the pro-apoptotic protein Bax and acts as a chaperone, binding only to activated and conformationally altered Bax (93). Bax was actually overexpressed in Jurkat-Tat101, as well as the BH3-domain only SOUL. SOUL has a similar function to Bax, inducing the mitochondrial membrane permeabilization and subsequent release of cytochrome c. The high expression of Bax in Jurkat-Tat101 could indicate a higher sensitivity to apoptosis. However, the pro-apoptotic function of Bax should be activated through the interaction with other factors as Bid or Bim, promoting a critical conformational change in Bax necessary for its death capacity (94, 95). In Jurkat-Tat101 cells, Bid was not efficiently cleaved to tBid, and the expression of Bim was reduced nearly 3-fold, indicating that the pro-apoptotic function of Bax was likely impaired by deficient activation. Moreover, Bim can be inhibited through its interaction with YWHAZ, a member of the 14-3-3 protein family that antagonizes the activity of pro-apoptotic proteins such as Bad, Bim, and Bax (96, 97). YWHAZ was enhanced in both Jurkat-Tat101 and Jurkat-Tat72, although it was heavily deamidated in Jurkat-Tat72, likely rendering a not fully functional protein, unable to control the pro-apoptotic proteins. Other anti-apoptotic factors such as PHB contributed to the higher protection against apoptosis provided by Tat101 but not Tat72. PHB is a potent survival factor that inhibits the release of cytochrome c and caspase-3 activation (98, 99), and it was increased 70-fold in Jurkat-Tat101, which was 2-fold higher than in Jurkat-Tat72.

Overall, these results indicated that the higher stability of the mitochondrial membrane integrity in Jurkat-Tat101 was due to a group of stabilizing proteins acting together to prevent the release of cytochrome c. A direct consequence of this inhibition was the inefficient activation of caspase-3, which could hinder the activation of other cellular pathways as the cell cycle control. In fact, DNA replication factors as MCM2 and MCM3 were overexpressed in Jurkat-Tat101. MCM3 is the substrate for caspase-3 cleavage, and the truncated forms contribute to initiate apoptosis (100), inactivating the MCM complex and preventing DNA replication (101). The low caspase-3 activity detected in Jurkat-Tat101 would contribute to MCM3 stability and therefore to the maintenance of the MCM complex, avoiding apoptosis. Other factors related to cell cycle progression, cytokinesis, and cell proliferation were also overexpressed in Jurkat-Tat101, such as NUDC, NASP, and PCNA (102–105). The overexpression of somatic NASP has been related to changes in NF-κB activity (106), which is known to be enhanced by Tat (23, 70). NF-κB may in turn activate the expression of several potent anti-apoptotic factors as those belonging to IAP family. The expression of XIAP was enhanced in both Jurkat-Tat101 and Jurkat-Tat72, but C-IAP2 was only overexpressed in Jurkat-Tat101, nearly 40 times. XIAP is a potent inhibitor of initiator caspases, caspase-9 (107), as well as execution caspases, caspases-3 and -7 (108), preventing both CD95- and Bax-induced apoptosis (109, 110). This would explain the high quantities of procaspase-3, -8, and -9 that were detected in Jurkat-Tat101 even after treatment with FasL. C-IAP2 is a component of the tumor necrosis factor receptor 2 (TNFR2) complex that inhibits cell death by direct repression of caspase activity (111), and it also targets for ubiquitin degradation several pro-apoptotic components of the TNF-α signaling pathway (112). Moreover, there should be a deregulation in the TNF signaling pathway because the expression of several components such as TNFRSF1A, Fas receptor, and TNF-β/lymphotoxin α showed an anomalous expression. Likely, the higher release of TNF cytokines by Jurkat-Tat cells was able to activate NF-κB via autocrine feedback, keeping a sustained activation of this transcription factor (113). As Tat72 lacks the motif KKKKVE, located in the second exon, which has been described as critical for NF-κB transactivation (70), the lower NF-κB basal activity detected in Jurkat-Tat72 regarding Jurkat-Tat101 should be related to this motif. Accordingly, Tat101 with mutated ESKKVE motif was less efficient than wild-type Tat101 to activate NF-κB and protect Jurkat against FasL-mediated apoptosis.

In summary, intracellular HIV-1 Tat deregulated the expression of several proteins related to apoptosis, rendering an anti-apoptotic general effect able to protect PBLs and Jurkat from FasL-induced apoptosis. This was achieved through the high stability of the mitochondrial membrane integrity because Tat101 increased the expression of several proteins, HSPs, NPM1, and PHB, committed to avoid mitochondrial depolarization, stabilize the cell cycle, and counteract pro-apoptotic factors such as Bid, Bim, or Bax. This effect was also achieved...
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FIGURE 9. Model summarizing the steps in Fas apoptotic pathway that were thwarted by the intracellular expression of Tat. See Table 1, Table 2, and text for further explanation.

through the ability of Tat101 to activate NF-κB, an essential transactivating factor that promotes cell survival through different mechanisms, such as the up-regulation of C-IAP2, XIAP, BCL2, and c-FLIPs. As Tat72 lacks the essential motif ESKK-KVE for activating NF-κB, this could be the most important mechanism through which Tat101 exerts its protective function. A scheme of the most important factors interfering with the apoptotic pathway in T lymphocytes, for which expression was modified by Tat, is depicted in Fig. 9.

In conclusion, Tat101-mediated protection against apoptosis would allow a prolonged HIV-1 production and spread and might explain why apoptosis mostly occurs in bystander uninfected cells. This viral strategy would be mainly achieved through the second exon. A better understanding of the molecular mechanisms responsible for the resistance to apoptosis in HIV-infected T cells is essential to fully characterize the ability of HIV-1 to establish a long-term infection.

Acknowledgments—We greatly appreciate the secretarial assistance of Olga Palao. We thank Centro de Transfusiones from Comunidad de Madrid, Spain, for providing the buffy coats.

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