NF-κB and p53 Are the Dominant Apoptosis-inducing Transcription Factors Elicited by the HIV-1 Envelope

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Abstract
The coculture of cells expressing the HIV-1 envelope glycoprotein complex (Env) with cells expressing CD4 results into cell fusion, deregulated mitosis, and subsequent cell death. Here, we show that NF-κB, p53, and AP1 are activated in Env-elicited apoptosis. The nuclear factor κB (NF-κB) super repressor had an antimitotic and antiapoptotic effect and prevented the Env-elicited phosphorylation of p53 on serine 15 and 46, as well as the activation of AP1. Transfection with dominant-negative p53 abolished apoptosis and AP1 activation. Signs of NF-κB and p53 activation were also detected in lymph node biopsies from HIV-1–infected individuals. Microarrays revealed that most (85%) of the transcriptional effects of HIV-1 Env were blocked by the p53 inhibitor pifithrin-α. Macroarrays led to the identification of several Env-elicited, p53-dependent proapoptotic transcripts, in particular Puma, a proapoptotic “BH3-only” protein from the Bcl-2 family known to activate Bax/Bak. Down modulation of Puma by antisense oligonucleotides, as well as RNA interference of Bax and Bak, prevented Env-induced apoptosis. HIV-1–infected primary lymphoblasts up-regulated Puma in vitro. Moreover, circulating CD4+ lymphocytes from untreated, HIV-1–infected donors contained enhanced amounts of Puma protein, and these elevated Puma levels dropped upon antiretroviral therapy. Altogether, these data indicate that NF-κB and p53 cooperate as the dominant proapoptotic transcription factors participating in HIV-1 infection.

Key words: Bax • mitochondria • NF-κB • Puma • Bak

Introduction
AIDS caused by HIV-1 involves the apoptotic destruction of lymphocytes (1–3). The envelope glycoprotein complex (Env) constitutes one of the major apoptosis inducers encoded by the HIV-1. The soluble Env derivative gp120 can induce apoptosis through interactions with suitable surface receptors (3, 4). HIV-1–infected cells that express mature Env (the gp120/gp41 complex) on the surface can also kill uninfected cells expressing the receptor (CD4) and the coreceptor (CXCR4 for lymphotropic Env variants, CCR5 for monocytotropic Env variants). This type of bystander killing is obtained by at least two distinct mechanisms. First, the two interacting cells (one that expresses Env and the other that expresses CD4 plus the coreceptor) may not fuse entirely and simply exchange plasma membrane lipids, after a sort of hemi-fusion process, followed by caspase-independent death (5). Second, the two cells can undergo cytoplasmic fusion (cytogamy), thus forming a syncytium, and undergo nuclear fusion (karyogamy) and apoptosis (6). In syncytia, the cyclin B1–dependent kinase 1 (Cdk1) is activated, a process that is required for karyogamy and that culminates in the mammalian target of rapamycin (mTOR)–mediated phosphorylation of p53 on serine 15. The transcriptional

Abbreviations used in this paper: Cdk1, cyclin B1–dependent kinase 1; DN, dominant-negative; Env, envelope glycoprotein complex; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HAART, highly active antiretroviral therapy; IKSR, IkB super-repressor; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB.
activation of p53 results in the expression of proapoptotic proteins, including Bax and cell death (7, 8). p53 and Bax have also been found to participate in the death of HIV–1–infected primary lymphoblasts (9, 10). Both the syncytium-dependent and -independent Env-induced apoptosis are suppressed by inhibitors of the gp41- and gp120-mediated coreceptor interactions (5, 6). Moreover, both are reduced by transfection with the Bax antagonist Bcl-2 (5, 6). In contrast, only the syncytium-dependent cell death can be blocked by inhibitors of Cdk1 (e.g., roscovitine), mTOR (e.g., rapamycin), or p53 (e.g., cyclic pifithrin-α; references 7, 8).

Some of the characteristics of syncytium–induced apoptosis, namely aberrant cyclin B expression (11), overexpression/activation of mTOR (7), phosphorylation of p53 on serine 15 (7, 11), and destabilization of mitochondrial membranes (6), also affect a subset of lymph node and peripheral blood lymphocytes from HIV–1–infected donors, correlating with viral load (7, 11–13). This suggests that the proapoptotic signal transduction pathway, which can be studied in Env–elicited syncytia, reflects, to some extent, the HIV–1–stimulated cell death, as observable among circulating lymphocytes. Based on this premise, we decided to further explore the proapoptotic signal transduction pathway participating in Env–induced syncytium–dependent cell killing. Therefore, we analyzed Env–elicited changes in the transcriptome and established the preponderant role of nuclear factor κB (NF-κB) as well as of p53 as proapoptotic transcription factors in HIV–1–induced cell death. Moreover, we identified Puma as a proapoptotic, p53–inducible protein that is critical for Env–induced apoptosis. The induction of Puma was not restricted to Env–elicited syncytia but was also found in Env–triggered syncytium–independent cell death in vitro. In addition, Puma expression was induced in HIV–1–infected patients.

**Materials and Methods**

**Cells and Culture Conditions for Apoptosis Induction.** HeLa cells stably transfected with the Env gene of HIV–1 LAI (HeLa Env) and HeLa cells transfected with CD4 (HeLa CD4) were cocultured at a 1:1 ratio (6) in DMEM supplemented with 10% FCS, l-glutamine, in the absence or presence of 1 μM roscovitine, 1 μM rapamycin (Sigma–Aldrich), 10 μM cyclic pifithrin–α, 5 μM MG132 (Calbiochem), the NF-κB inhibitory peptide SN50, or its negative control SN50M (20 μM; BIOMOL Research Laboratories, Inc.). HeLa/Env and HeLa/CD4 were cocultured at different ratios (6). U937 or Jurkat cells were cultured with 500 ng/ml recombinant gp120 protein.

**Plasmids, Transfection, and Transcription Factor Profiling.** For transcription factor profiling, HeLa CD4 cells were transfected with different luciferase constructs (Mercury™ pathway profiling system from CLONTECH Laboratories, Inc.; 2 μg DNA) using Lipofectamine 2000™ (2 μl; Invitrogen) 24 h before fusion with HeLa Env cells (25 × 10⁴ in 2 ml) and the luciferase activity was measured 24 h later using the luciferase reporter assay kit from CLONTECH Laboratories, Inc. Transfection with pcDNA3.1 vector only, WT, dominant-negative (DN) Cdk1 mutant (14), the IkB super-repressor (IκSR; a gift from L. Schmitz, University of Bern, Bern, Switzerland), p53 DN plasmids (p53H273; a gift from T. Soussi, Institut Curie, Paris, France), or a p53-responsive enhanced green fluorescent protein (GFP) plasmid (a gift from K. Wiman, Karolinska Hospital, Stockholm, Sweden) was performed 24 h before coculture of HeLa CD4 and HeLa Env cells. The frequency of GFP–expressing cells was assayed by cytometry on a FACS Vantage™ (Becton Dickinson) equipped with a 100–μm nozzle, allowing for the analysis of relatively large cells. Afterwards, cells were counterstained with 10 μg/ml Hoechst 33334 (15 min), and the FACS® gates were set on syncytia (i.e., cells with a DNA content >4 n) (11).

**Immunofluorescence and Cytometry.** Rabbit antisera specific for p53S15P, p53S46P (Cell Signaling Technology), or the NH₂ terminus of Bax (N20; Santa Cruz Biotechnology, Inc.) were used on paraformaldehyde (4% wt:vol) and picric acid–fixed (0.19% vol:vol) cells (15) and revealed with a goat anti–rabbit IgG conjugated to Alexa 568 (red) or Alexa 488 (green) fluorochromes obtained from Molecular Probes. Cells were also stained for the detection of the NH₂ terminus of Bak (Ab1; Oncogene Research Products), p53S15P (mAb), cyclin B1 (mAb; BD Transduction Laboratories), and IkBαS32/36P (mAb; Cell Signaling Technology) revealed by anti–mouse IgG Alexa conjugates. Cells were counterstained with Hoechst 33342, which allows discernment of karyogamy and apoptotic chromatin condensation (11).

**Immunoblots.** Samples were prepared from HeLa Env and HeLa CD4 single cells mixed at a 1:1 ratio in lysis buffer (single cells control) or from HeLa Env/CD4 syncytia. Protein samples from HeLa Env and U937 mixed at different ratios were also prepared and subjected to immunoblot analysis. Aliquots of protein extracts (40 μg) were subjected to immunoblotting using antibodies specific for IkBαS32/36P, p53S15P, p53S46P, p53 (Cell Signaling Technology), Puma (rabbit anti–human Puma antisera; United States Biological or Orbigene; and mouse anti–human Puma antibody; Upstate Biotechnology; all gave similar results), β-tubulin (mAb; Sigma–Aldrich), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, mAb; Chemicon).

**Microarrays, Macroarrays, and Quantitative RT–PCR.** HeLa CD4 and HeLa Env cells were labeled with CellTracker™ red or CellTracker™ green (15 μM, 30 min at 37°C), respectively, and washed extensively before coculture. After 18 or 36 h of coculture, in the presence or absence of 10 μM cyclic pifithrin–α (readed once upon 24 h), the cells were subjected to FACS® purification of syncytia (which are double positive) or single cells, as described previously (11). mRNA preparations were obtained with the RNeasy Mini kit (Qiagen), quality–controlled with a bioanalyzer (model 2100; Agilent Technologies), alternatively labeled with Cy3/Cy5 fluorochromes, mixed for pairwise comparisons, and hybridized to 19-k human arrays. Arrays were scanned with a confocal laser scanner (ScanArray model 4000; Perkin–Elmer), quantified with software (QuantArray; PerkinElmer) at the Ontario Cancer Institute Microarray Center (http://www.microarrays.ca), and analyzed with GeneTraffic software (Lobion Informatics). All experiments were performed in triplicates, while switching the Cy3/Cy5 labeling (six data points for each pair-wise comparison) and up-regulations by 50% or down-regulations by 30% were listed. Data showing the transcriptional modification of genes with unknown function are listed in Table SI (available at http://www.jem.org/cgi/content/full/jem.20031216/DC1). Macroarrays were performed using a TranSignal™ p53 target gene array (Ponomics) and quantified using the National Institutes of Health image software. For quantitative RT–PCR, cDNAs were synthesized from 1 μg of total RNA with reverse transcriptase MuLV (Roche). The TaqMan universal PCR was performed on.
Results

Transcription Factor Profiling of HIV-1 Env-elicted Apoptosis.

In a model culture system of syncytium-dependent cell death, HeLa cells stably transfected with the Env gene from HIV-1LAI/IIIb (HeLa Env) were fused by coculture with CD4/CXCR4-expressing HeLa cells (HeLa CD4; references 6, 7, 17, 18). Syncytium-induced transcriptional effects were monitored by transfecting each of the two cell lines with a series of luciferase reporter gene constructs containing promoter elements responsive to a series of different transcription factors. This approach revealed that, among a panel of 12 different transcription factors, only NF-κB, p53, and AP1 were activated by the Env–dependent syncytium formation (Fig. 1A). To determine the hierarchy among these transcription factors, cells were cotransfected with the aforementioned luciferase reporter constructs as well as a nonphosphorylatable mutant of IkB (IKSR; reference 19) or with a DN mutant of p53 (p53H273; reference 20). Both IKSR and p53H273 abrogated the increase of NF-κB, p53, and AP1-dependent transcription found in syncytia (Fig. 1B). These data point to an obligatory cooperation between NF-κB and p53, upstream of AP1, in Env–elicited syncytia.

NF-κB Is Required for Env-elicted Mitosis and p53 Activation.

NF-κB is usually considered as an antia apoptotic transcription factor (21, 22). Intrigued by its putative implication in syncytial apoptosis, we further investigated the possible implication of NF-κB in syncytial apoptosis. Cytoplasmic fusion is followed by nuclear fusion (karyogamy) accompa-
nying an aberrant, cyclin B1-dependent entry into mitosis (11, 18). This allows for the distinction of early (prekaryogamic) and late (karyogamic) syncytia. A significant fraction of prekaryogamic syncytia exhibited the phosphorylation of IκB on serine residues 32 and 36 (IκBBS32/36P), as detectable with a phosphoepitope-specific antibody. IκBBS32/36P was found in the cytoplasm of prekaryogamic syncytia. In karyogamic syncytia, IκBBS32/36P was confined to the nucleus (Fig. 2 A). p53 was found to exhibit an activation-associated phosphorylation pattern, both on serine 15 (Fig. 2 B, p53S15P) and serine 46 (Fig. 2 C, p53S46P), only in karyogamic syncytia. Immunoblot analysis confirmed the phosphorylation of IκB and p53 (Fig. 2 D). Kinetic analysis confirmed a precise order of phosphorylation events (IκBBS32/36P → p53S15P → p53S46P) (Fig. 2 E).

Transfection of HeLa CD4 and HeLa Env cells with a p53-inducible GFP construct allowed for the quantitative assessment of p53-dependent transcriptional effects within syncytia. p53-dependent transcription was strongly inhibited by transfection with IKSR (Fig. 3 A), as well as by pharmacological inhibition of the NF-κB translocation with SN50 (Fig. 3 B). Simultaneously, both IKSR (Fig. 3 C) and SN50 (Fig. 3 D) inhibited the emergence of karyogamic syncytia, suggesting that NF-κB is required for mitotic progression of syncytia. Indeed, NF-κB inhibition fully prevented the syncytial accumulation of cyclin B1, as determined by quantitative immunofluorescence analysis (Fig. 3 E). Inhibition of the Cdk1 by DN Cdk1 (Fig. 3 A) or roscovitine (Fig. 3 B) inhibited the p53–dependent transcription, while inhibiting karyogamy (Fig. 3, C and D). Altogether, these findings indicate that NF-κB is essential for the aberrant, cyclin B1/Cdk1–mediated entry into karyogamy that occurs upstream of p53 activation. The repression of NF-κB reduced the karyogamy–associated phosphorylation of p53 (both p53S15P and p53S46P). Moreover, transfection with DN p53 partially inhibited the phosphorylation of IκB and totally blocked its translocation into the nucleus (Fig. 3 E). These findings point again to an obligate cross-talk between p53 and NF-κB.

IκB phosphorylation was also detectable in lymph nodes from HIV-1 carriers (but not in those from uninfected controls), mainly among mitotic cells (Fig. 4 B). Moreover, the phosphorylation of p53S46P was induced by HIV-1 infection of primary CD4+ lymphoblasts (see below) and was...
detectable in lymph node biopsies from HIV-1 carriers (Fig. 4 A). Altogether, these data extend the notion of HIV-1–induced NF-κB activation and p53 activation.

**HIV-1 Env-elicited Transcriptional Effects Involve p53 and Lead to Puma Overexpression.** To further explore the implication of transcription factors in Env-induced syncytial apoptosis, microarrays were performed on HeLa Env/CD4 cocultures maintained for 18 or 36 h in the presence or absence of cyclic pifithrin-α, a chemical inhibitor of p53 or p53, and apoptotic parameters. Syncytia were generated as in A, after transfection with pcDNA3.1 (Control), IKSR, or p53, followed by immunofluorescence detection of cyclin B1. The frequency of syncytia exhibiting an increase in cyclin B immunostaining was determined in three independent experiments after 24 h of coculture. Moreover, the frequency of syncytia exhibiting positive cytoplasmic or nuclear IkB53/36P staining, p53S15P or p53S46P, mitochondrial cytochrome c release, or nuclear apoptosis was determined. Asterisks indicate significant inhibitory effects (P < 0.01; paired Student's t test; mean ± SD; n = 3).

![Figure 3](image-url)  
Rules governing the activation of p53 in Env-elicited syncytia. (A) Genetic inhibition of p53-dependent transcription. HeLa CD4 cells were cotransfected with a p53-inducible GFP construct, together with vector only (pcDNA3.1), a dominant-negative (DN) Cdk1 mutant, DN p53 (p53H273), or IKSR. After 24 h, the cells were either left alone (single cells) or cocultured with HeLa Env cells for 36 h, followed by cytofluorometric determination of GFP. (B) Pharmacological inhibition of p53-dependent transcription. HeLa CD4 cells were transfected with a p53-inducible GFP construct. After 24 h, cells were cultured in the presence of the indicated agents, in the presence or absence of HeLa Env cells for 24 h, followed by determination of the frequency of GFP-expression single cells or syncytia. (C) Inhibition of karyogamy by IKSR. HeLa CD4 cells were transfected with the indicated constructs as in A, and the frequency of cells exhibiting nuclear fusion was scored upon coculture with HeLa Env cells. (D) Inhibition of karyogamy by NF-κB inhibitors. Syncytia were treated with various drugs as in B, and the frequency of karyogamic cells was scored. (E) Effect of IKSR and DN p53 on cyclin B1, karyogamy, phosphorylation of IkB or p53, and apoptotic parameters. Syncytia were generated as in A, after transfection with pcDNA3.1 (Control), IKSR, or DN p53, followed by immunofluorescence detection of cyclin B1. The frequency of syncytia exhibiting an increase in cyclin B immunostaining was determined in three independent experiments after 24 h of coculture. Moreover, the frequency of syncytia exhibiting positive cytoplasmic or nuclear IkB53/36P staining, p53S15P or p53S46P, mitochondrial cytochrome c release, or nuclear apoptosis was determined. Asterisks indicate significant inhibitory effects (P < 0.01; paired Student's t test; mean ± SD; n = 3).

![Figure 4](image-url)  
Phosphorylation of IkBα and p53 in lymph nodes from HIV-1 carriers. Lymph nodes from age- and sex-matched controls or untreated HIV-1 carriers were stained for the immunocytochemical detection (red) of IkBα (S32/36)P (A) and p53S46P (B). Nuclei are counterstained in blue. Similar results indicating a scattered p53S46 staining and staining of mitotic cells with IkBα(S32/36)P were found in three independent asymptomatic, untreated HIV-1–infected donors with a viremia of >10^5 copies/ml.

**Figure 4.** Phosphorylation of IkBα and p53 in lymph nodes from HIV-1 carriers. Lymph nodes from age- and sex-matched controls or untreated HIV-1 carriers were stained for the immunocytochemical detection (red) of IkBα (S32/36)P (A) and p53S46P (B). Nuclei are counterstained in blue. Similar results indicating a scattered p53S46 staining and staining of mitotic cells with IkBα(S32/36)P were found in three independent asymptomatic, untreated HIV-1–infected donors with a viremia of >10^5 copies/ml.

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Perfettini et al. 633
Table I. Classification of Modulated Genes with Known Function in Env-induced Syncytia

| Accession no. | Symbol | Gene function | 18 h | 36 h |
|---------------|--------|---------------|------|------|
|               |        |               | –    | +    |
|               |        |               | –    | +    |

**Cell cycle**

- W09443 HMG14 high-mobile group (nonhistone chromosomal) protein 14
  - <1.5
  - 1.54 ± 0.06
  - 1.73 ± 0.11
  - <1.5
- AA1475 GAPCENA rab6 GTase activating protein (GAP and centrosome associated)
  - >0.67
  - >0.67
  - 0.61 ± 0.01
  - >0.67
- W90163 RAMP RA-regulated nuclear matrix-associated protein
  - >0.67
  - >0.67
  - 0.45 ± 0.14
  - >0.67
- R10158 L0CS118S protein × 0091/membrane of the ATP-dependent serine protease (LON) family
  - 1.58 ± 0.08
  - <1.5
  - <1.5
  - <1.5
- H66120 CENPE centromere protein E (312 kD)
  - 3.07 ± 0.78
  - 3.55 ± 0.43
  - 7.39 ± 2.39
  - 7.01 ± 0.27

**DNA repair**

- W37869 EZH1 enhancer of zeste (Drosophila melanogaster) homologue 1
  - 0.55 ± 0.10
  - >0.67
  - >0.67
  - >0.67

**Tumor suppressor/apoptosis**

- R19586 PPL15 protocadherin protein 1 (Peltzenu-Merzbacher disease)
  - 1.736 ± 60.26
  - <1.5
  - 2.42 ± 0.15
  - 3.84 ± 1.57
- R80461 PDICD5 programmed cell death 5
  - <1.5
  - <1.5
  - 1.82 ± 0.13
  - 2.53 ± 0.8
- H28623 CREG cellular repressor of E1A-stimulated genes
  - >0.67
  - >0.67
  - 0.51 ± 0.23
  - >0.67
- N80014 BRAP BRCA1-associated protein
  - 0.14 ± 0.1
  - >0.67
  - >0.67
  - >0.67
- R77391 RECK reversion-inducing cysteine-rich protein with Kazal motifs
  - 0.44 ± 0.04
  - >0.67
  - >0.67
  - >0.67

**Receptor/growth factor regulator**

- AA088248 FGRF1 fibroblast growth factor receptor 1
  - <1.5
  - <1.5
  - 1.62 ± 0.03
  - <1.5
- W38478 IGFBP7 insulin-like growth factor binding protein 7
  - <1.5
  - <1.5
  - 1.6 ± 0.07
  - <1.5
- AA046958 IGFBP1 insulin-like growth factor binding protein 1
  - >0.67
  - >0.67
  - 0.63 ± 0.05
  - >0.67
- T39448 ADRA2C adrenergic α-2C receptor
  - 1.90 ± 0.31
  - 1.77 ± 0.28
  - 3.24 ± 0.56
  - 3.95 ± 1.24
- N30625 CORT cortistatin
  - >0.67
  - >0.67
  - 0.6 ± 0.1
  - >0.67
- AA151595 IFNAR2 interferon (α, β, and γ) receptor 2
  - 1.78 ± 0.28
  - <1.5
  - <1.5
  - <1.5

**Signaling**

- H77460 LNk lymphocyte adenosine receptor protein
  - <1.5
  - <1.5
  - 2.46 ± 0.56
  - <1.5
- R14326 HERC1 hect domain and RCC1 (CHC1)-like domain (RLD) 1
  - <1.5
  - <1.5
  - 2.78 ± 0.43
  - 4.46 ± 1.82
- H18190 JAK1 Janus kinase 1
  - <1.5
  - <1.5
  - 1.79 ± 0.19
  - 2.87 ± 0.86
- AA037843 NET-7 transmembrane 4 superfamily member (tetraspan NET-7)
  - >0.67
  - >0.67
  - 0.52 ± 0.08
  - >0.67
- H56744 CC1.3 spliceosome factor, coactivator of activating protein-1, and estrogen receptors
  - 2.13 ± 0.3
  - 2.08 ± 0.64
  - 2.86 ± 0.15
  - 3.23 ± 0.82
- R17538 PAIP2C4 poly(A)-binding protein, cytoplasmic 4 (inducible form)
  - 1.88 ± 0.25
  - 1.91 ± 0.19
  - 2.08 ± 0.43
  - 2.48 ± 0.78
- R44875 ARIP1 atrophin-1 interacting protein 1
  - 1.90 ± 0.33
  - 2.34 ± 0.4
  - 4.58 ± 1.19
  - 4.99 ± 0.56
- AA040497 ZFXH1B zinc finger homeobox 1B/encoding Smad-interacting protein 1
  - >0.67
  - >0.67
  - 0.48 ± 0.16
  - >0.67
- H62028 Dyrk3 dual-specificity tyrosine-(Y)- phosphorylation regulated kinase 3
  - >0.67
  - >0.67
  - 0.40 ± 0.35
  - >0.67
- N45959 AKAP6 A kinase (PRKA) anchor protein 6
  - >0.67
  - >0.67
  - 0.60 ± 0.03
  - >0.67
- AA135745 CAMK2G calcium/calmodulin-dependent protein kinase (CaM kinase) II γ
  - 0.5 ± 0.1
  - >0.67
  - >0.67
  - >0.67

**Metabolism/protein degradation**

- H47026 MGA3 mammalian (β-1,4-)glycoprotein family member 14-N-acetylglucosaminyltransferase
  - <1.5
  - 2.32 ± 0.17
  - 2.31 ± 0.43
  - <1.5
- H60458 ACOX2 acyl-coenzyme A oxidase 2, branched chain
  - <1.5
  - 1.62 ± 0.03
  - 1.63 ± 0.07
  - <1.5
- T91335 LUC7L LUC7L (Saccharomyces cerevisiae)-like
  - 1.72 ± 0.05
  - <1.5
  - 2.43 ± 0.31
  - 2.67 ± 0.55
- AA099341 GBF1 Golgi-specific brefeldin A resistance factor 1
  - 0.54 ± 0.05
  - >0.67
  - >0.67
  - >0.67
- W17311 SDHIB succinate dehydrogenase complex, subunit B, iron sulfur (l) p
  - 1.99 ± 0.4
  - 2.48 ± 0.80
  - 4.41 ± 0.67
  - <1.5
- AA115377 SLC2A10 solute carrier family 2 (facilitated glucose transporter), member 10
  - 0.56 ± 0.13
  - >0.67
  - >0.67
  - >0.67
- N48749 FAC13 fatty-acid-coenzyme A ligase, long-chain 3
  - 0.61 ± 0.04
  - >0.67
  - >0.67
  - >0.67
- W39228 CTSG cathepsin G
  - <1.5
  - <1.5
  - 4.2 ± 1.57
  - 5.86 ± 0.47
- W59301 GCSH glycine cleavage system protein H (aminomethyl carrier)
  - 0.61 ± 0.04
  - >0.67
  - >0.67
  - >0.67
- W79652 ATE1 arginyltransferase 1
  - 1.72 ± 0.33
  - 2.08 ± 0.22
  - 2.36 ± 0.21
  - 2.79 ± 1.03
- H98809 PABPC1 poly(A)-binding protein cytoplasmic 1
  - >0.67
  - >0.67
  - 0.58 ± 0.04
  - >0.67
- R81846 FTL ferritin, light polypeptide
  - >0.67
  - >0.67
  - 0.60 ± 0.05
  - >0.67

The list contains genes of known function from syncytia obtained after 18 or 36 h of coculture, in the presence or absence of 10 µM cyclic pthrin-α. Compared with single cells, only genes that showed an induction or inhibition of mRNA expression (by a factor of ±1.5 or ±0.67, respectively) were considered. Changes in mRNA expression were determined by microarrays as described in Materials and Methods.
Perfettini et al. confirmed the induction of Puma and Bax (and in particular the dominant Bax-α splice variant) transcripts (Fig. 6 A), which is consistent with the fact that syncytia overexpress Bax (7) and Puma (Fig. 6 B) at the protein level.

Up-regulation of Puma Is Required for Syncytial Apoptosis. Puma has been identified recently as a p53-inducible “BH3-only” protein that can activate the proteins Bax and Bak to adopt a proapoptotic conformation (with exposure of the NH₂ terminus), to insert into the outer mitochondrial membrane and to stimulate the release of cytochrome c, leading to subsequent caspase activation and apoptosis (23–25). Accordingly, transfection with antisense oligonucleotides designed to down-regulate the expression of Puma (Fig. 7 B, inset; reference 24) inhibited the exposure of the NH₂ termini of Bax and Bak (as detected with conformation-specific antibodies), the release of cytochrome c from syncytial mitochondria (as detected by immunofluorescence staining), and the apoptosis-associated chromatin condensation (Fig. 7 A and B). Inhibition of NF-κB with IKSR (Fig. 7 C) or SN50, as well as inhibition of p53 with cyclic pifithrin-α (Fig. 7 D) all inhibited the activation of Bax and Bak. RNA interference of Bax or Bak, the two Puma receptors, significantly reduced syncytal apoptosis, as indicated by suppression of the nuclear chromatin condensation and the mitochondrial cytochrome c release (Fig. 7 E). As a side observation, we found that RNA interference of Bak prevented the exposure of the NH₂ terminus of Bax but not vice versa, suggesting a hitherto unsuspected hierarchy among these proapoptotic proteins. Altogether, the data suggest that Puma acts on Bax and/or Bak to kill syncytia in a p53-dependent fashion.

Induction of Puma in Several Models of HIV-1 and Env-induced Apoptosis In Vitro. When cocultured with HeLa Env cells, U937 cells (which lack functional p53; reference 26) do not form heterokarya, but rather undergo apoptosis after a transient cell-to-cell interaction (5, 6). As shown in Fig. 8 A, apoptosis correlates with the induction of Puma, and the up-regulation of Puma is not inhibited by cyclic pifithrin-α, underscoring that Puma can be induced in a p53-independent fashion. Similarly, different recombinant gp120 variants (MN and 93TH975 with preference for CXCR4; BaL and SF162 with preference for CCR5) induced cell death and Puma protein expression in U937 cells (which express both CXCR4 and CCR5), correlating with the induction of PUMA (Fig. 8 B). Transfection of U937 cells with p53 accelerated the kinetics of gp120-induced Puma induction at early time points when gp120 alone still had no Puma-inducing effect (Fig. 8 C). Thus, p53-independent and -dependent pathways can cooperate.
to cause Puma induction. In p53-sufficient Jurkat cells (27), which only express CXCR4, only the R4-tropic gp120 variants induced apoptosis and Puma expression (Fig. 8 D). This effect was strongly inhibited by cyclic pifithrin-α (Fig. 8 E). Primary CD4+ lymphoblasts from healthy donors infected with HIV-1LAI/IIIb also manifested the induction of Puma, at the protein level, well after the phosphorylation of p53 (Fig. 8 F). CD4+ lymphoblasts infected with clinical HIV-1 isolates (16) manifested the induction of Puma, which could be detected in yet viable syncytia (Fig. 8 G). Thus, Env and HIV-1 infection induce Puma expression in a variety of experimental systems.

Enhanced Puma Expression in HIV-1-infected Patients. Lymph nodes from untreated HIV-1 patients with high HIV-1 titers (≥20,000 copies/ml) stained more positively for Puma than did biopsies from uninfected controls (Fig. 9 A). Using immunocytochemical methods, Puma+ cells were found among all relevant (CD4+, CD8+, and CD14+) cells from HIV-1-infected donors with such high HIV-1 titers, with a preference for CD4+ and CD14+ cells (Fig. 9 B). Puma expression levels among the PBMCs correlated positively with HIV-1 titers (Fig. 9 C). The enhanced Puma expression could also be detected by immunoblotting among circulating CD4+ cells from naive (nontreated) HIV-1 patients as compared with healthy controls or HAART-treated individuals (Fig. 9 D). Moreover, longitudinal studies revealed that Puma levels were reduced to normal levels when patients were undergoing successful antiretroviral therapy (Fig. 9 E), in line with the well-established fact that antiretroviral therapy reduces the
increased spontaneous apoptosis of circulating CD4+ lymphocytes from HIV-1 carriers (28, 29). These data establish that productive HIV infection is associated with the overexpression of the apoptotic effector Puma.

**Discussion**

Our data strongly indicate that one of the dominant transcription factors activated in Env-elicited syncytia is p53. More than 80% of the genes whose expression level changed did so via a p53-dependent mechanism, as indicated by the fact that chemical p53 inhibition abolished this change. Inhibition of p53 prevented the expression of several proapoptotic genes and disabled the death program. p53 appears to be activated through at least two phosphorylation events, which are both detectable in HIV-1 carriers. One affects serine 15 and is mediated by mTOR, whereas the other affects serine 46. This latter phosphorylation event is well known to enhance the apoptogenic potential of p53 and to relay to mitochondrial apoptosis (30, 31). Although the nature of the kinase acting on serine 46 (which is not mTOR; not depicted) remains to be determined, it appears clear that this phosphorylation event occurs downstream of the Cdk1-dependent karyogamy. Genetic or pharmacological inhibition of NF-κB prevented the cyclin B-dependent Cdk1 activation, as well as the phosphorylation of p53 on both serine 15 and 46. Thus, the proapoptotic cooperation between NF-κB and p53 observed in this particular model is likely to be indirect, via a cell cycle–related effect of NF-κB. This would be consistent with the fact that NF-κB is mostly viewed as an apoptosis-inhibiting transcription factor (21). NF-κB activation would be necessary for the advancement in cell cycle, which enables p53-dependent transcription and apoptosis, yet would have no direct proapoptotic transcriptional effects. Indeed, there is no indication for the induction of antiapoptotic NF-κB target genes in our system (Tables I and Table S1). In contrast, the p53-mediated expression of Puma appears to be direct, at least in Env-induced syncytia (Fig. 1–6). It is important to note that Puma expression is not under the exclusive control of p53, at least in transgenic mice (32), and that p53-independent pathways may contribute to the Env-elicited Puma expression (Fig. 8 A–C) and actually cooperate with the p53-dependent ones (Fig. 8 C). Thus,

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**Figure 8.** Induction of Puma by Env and HIV-1 in vitro. (A) Induction of Puma in U937 cells cocultured with HeLa Env cells. U937 cells were mixed at different ratios with HeLa Env cells just before the preparation of cell lysates for immunoblot detection of Puma [Control] or coculture for 36 h in the absence or presence of the p53 inhibitor cyclic pifithrin-α (5 μM). The percentage of dead U937 cells was determined by trypan blue exclusion. (B) Induction of Puma by recombinant gp120 protein in U937 cells. U937 were exposed to the indicated gp120 protein [at 500 ng/ml] and cell death, Puma, and GAPDH expression were determined after 4 d. (C) Cooperation between gp120 and p53 to induce Puma in U937 cells. Cells were mock transfected or transfected with wild-type p53. 1 d later, the indicated gp120 proteins were added, and the expression of 33 and Puma were determined 48 h later. (D) Induction of Puma by recombinant gp120 protein in Jurkat cells as in B. (E) Effect of cyclic pifithrin-α. Jurkat cells were treated for 4 d with 500 ng/ml gp120 protein and/or 10 μM cyclic pifithrin-α, followed by determination of Puma expression. (F) Induction of Puma by infection of primary lymphoblasts in vitro. CD4+ lymphoblasts from a healthy donor were infected with HIV-1 LAI/IIIb for the indicated period, and proteins (40 μg/lane) were subjected to immunoblot determination of p53 phosphorylation and Puma expression. (G) Puma induction in CD4+ lymphoblasts from a healthy donor infected with a clinical HIV-1 isolate. 5 d after infection, cells were subjected to immunohistochemical detection of Puma. Uninfected cells served as a negative control. Results typical for five independent experiments are shown.
different pathways (p53-dependent or -independent) may contribute to the induction of Puma, in vivo, in the HIV-1–infected patient. That at least part of this Puma-inducing effect involves p53 is suggested by the fact that p53 does manifest proapoptotic posttranscriptional modifications (in particular, the phosphorylation of serine 46), which are detectable in resident and circulating immune cells from HIV-1 carriers.

A cornucopia of mechanisms contribute to the enhanced apoptotic decay of T lymphocytes in HIV-1 infection. Rather than viewing the virus-mediated and host-dependent contribution to lymphodepletion in an exclusive fashion, it is conceivable that proapoptotic mechanisms actually cooperate in the patient (1–3). As shown here, both soluble and membrane-bound Env can induce the expression of the proapoptotic protein Puma, a protein that acts on mitochondria to trigger apoptosis (23–25). Recently, it has also been shown that a mutation in Vpr that reduces its apoptogenic potential (R77Q) is found in ∼80% of long-term nonprogressors; that is, patients with high HIV-1 titers yet normal CD4 counts and normal levels of spontaneous apoptosis (33, 34). Vpr directly acts on mitochondria, on a molecular complex formed by Bax, and on the adenine nucleotide translocase (33, 35), and can also elicit p53-dependent transcriptional effects (36). Although the in vitro systems characterized here were devoid of Vpr, these findings illustrate that the Env- and Vpr-elicited apoptotic pathways could cooperate in stimulating p53- and mitochondrion-mediated cell death. Such cooperative effects would be compatible with previous observations indicating a reduced expres-
sion of Bcl-2 in rarified CD4+ cells (37) and an enhanced level of Bax protein in those CD8+ cells that are undergoing apoptosis (38). Indeed, Bcl-2 is known to be down-regulated by p53 (39), whereas Bax is up-regulated (40). The p53-induced imbalance in the precarious equilibrium of pro-apoptosis (38). Indeed, Bcl-2 is known to be down-regulated (40). The p53-induced imbalance in the precarious equilibrium of pro-animal Contre le Cancer, as well as grants from ANRS, FRM, the National Contre le Cancer, as well as grants from ANRS, FRM, the National Institute of Allergy and Infectious Diseases Research and Reagents Program for cell lines and gp120 protein, Mr. S. Showalter and Ms. M. Garcia-Moll (BioMolecular Technology) for gp120 from HIV-193TH975.

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639 Perfettini et al.
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