

Essential role of p53 phosphorylation by p38 MAPK in apoptosis induction by the HIV-1 envelope

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The proapoptotic activity of the transcription factor p53 critically depends on the phosphorylation of serine 46 (p53S46P). Here, we show that syncytia containing p53S46P could be detected in lymph node biopsies from human immunodeficiency virus (HIV)-1 carriers, in the brain of patients with HIV-1-associated dementia and in cocultures of HeLa expressing the HIV-1 envelope glycoprotein complex (Env) with HeLa cells expressing CD4. In this latter model, cell death was the result of a sequential process involving cell fusion, nuclear fusion (karyogamy), phosphorylation of serine 15 (p53S15P), later on serine 46 (p53S46P), and transcription of p53 target genes. Cytoplasmic p38 mitogen-activated protein kinase (MAPK) was found to undergo an activating phosphorylation (p38T180/Y182P [p38 with phosphorylated threonine 180 and tyrosine 182]) before karyogamy and to translocate into karyogamic nuclei. p38T180/Y182P colocalized and coimmunoprecipitated with p53S46P. Recombinant p38 phosphorylated recombinant p53 on serine 46 *in vitro*. Inhibition of p38 MAPK by pharmacological inhibitors, dominant-negative p38, or small interfering RNA, suppressed p53S46P (but not p53S15P), the expression of p53-inducible genes, the conformational activation of proapoptotic Bax and Bak, the release of cytochrome c from mitochondria, and consequent apoptosis. p38T180/Y182P was also detected in HIV-1-induced syncytia, *in vivo*, in patients' lymph nodes and brains. Dominant-negative MKK3 or MKK6 inhibited syncytial activation of p38, p53S46P, and apoptosis. Altogether, these findings indicate that p38 MAPK-mediated p53 phosphorylation constitutes a critical step of Env-induced apoptosis.

Viral infection can result into apoptosis, in particular at late stages of the viral life cycle when viral spreading and/or subversion of the host's immune system can serve the virus' purpose. In accord with this general rule, HIV-1 encodes for a variety of different proteins that can induce apoptosis (1–3). To reveal the apoptogenic effect of some, clinically important HIV-1-encoded protein such as Vpr (4), it is required to take advantage of so called pseudotyped viruses, that is genetically modified HIV-1 strains in which the endogenous envelope glycoprotein complex (Env) gene has been replaced by nonapoptogenic Env proteins from other viruses (4, 5). This underscores the notion that Env is, at least *in vitro*, the principal apoptosis-inducing protein encoded by HIV-1 (6–9).

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The Env glycoprotein precursor protein (gp160) undergoes proteolytic maturation to gp41 (membrane inserted) and gp120 (membrane inserted or shed from the cell surface). Soluble gp120 can stimulate proapoptotic signal via an action on chemokine receptors (CXCR4 for lymphotropic Env variants, CCR5 for monocytoprotropic Env variants; 9–11), pertussis toxin-sensitive G proteins (11), the p38 mitogen-activated protein kinase pathway (12), and/or a rapid cytosolic Ca²⁺ increase (13). The membrane-bound gp120–gp41 complex expressed on the surface of HIV-1-infected cells can induce apoptosis via interaction with uninfected cells expressing the receptor (CD4) and the chemokine coreceptor CXCR4. Although this interaction can signal for apoptosis via a transient cell-to-cell contact (14), in most instances, this interaction induces cellular fusion

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Abbreviations used: ATF-2T271P, ATF-2 with phosphorylated tyrosine 271; Cdk1, cyclin-dependent kinase-1; DN, dominant-negative; Env, envelope glycoprotein complex; GFP, green fluorescent plasmid; HIP, homeodomain-interacting protein; MKK3/6 S189/207P, MKK3 with phosphorylated serine 189 and/or MKK6 with phosphorylated serine 207; mTOR, mammalian target of rapamycin; p38T180/Y182P, p38 with phosphorylated threonine 180 and tyrosine 182; p53S15P, p53 with phosphorylated serine 15; p53S46P, p53 with phosphorylated serine 46; PEG, polyethylene glycol.

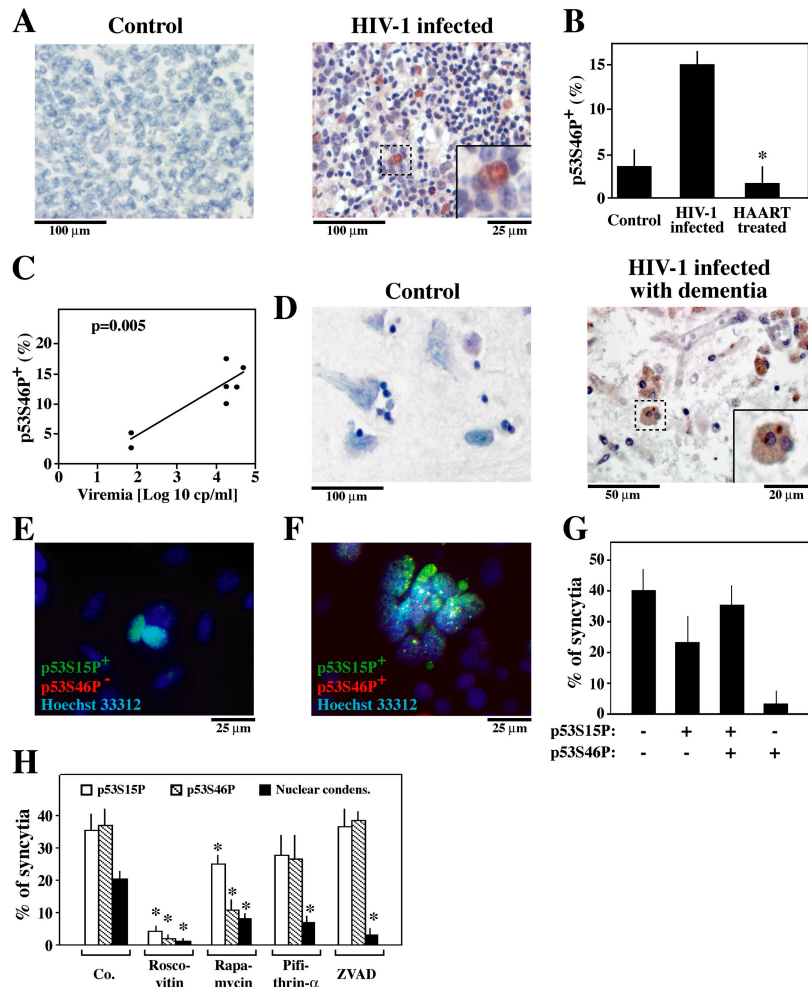


Figure 1. Phosphorylation of p53 on S46 (p53S46P) in HIV-1 infection in vivo and Env-elicited syncytium formation in vitro.

(A) p53S46P in lymphoid tissue. Lymph node biopsies from an untreated HIV-1-infected patient or an uninfected individual (control) were stained with an antiserum recognizing p53S46P. The insert shows a bona fide syncytium positive for p53S46P. Note that the percentage of p53S46P+ cells in HIV-1-infected, untreated individuals was 75 ± 12 (x + SEM, $n = 10$) in the T cell area, it was <1% in uninfected controls ($n = 5$). (B) p53S46P+ PBMC, as determined by immunocytochemistry on PBMC from uninfected controls ($n = 5$), untreated patients with viral loads $>10^5$ copies/ml ($n = 8$) and patients under HAART with undetectable virus titers ($n = 10$). Results are means \pm SD. (C) Correlation between the frequency of p53S46P+ PBMC and viral load among untreated HIV-1 carriers. (D) p53S46P in multinuclear cells from the frontal cortex with HIV-1-associated dementia. Note that such p53S46P+ cells were not found in control brains (from noninfected individuals, $n = 10$), yet were found in 17 out of 17 cases of HIV-1-associated dementia. (E and F) p53S15P and p53S46P in syncytia

(cytogamy; 6, 7, 15) followed by nuclear fusion (karyogamy) within the syncytium (16). This nuclear fusion is the expression of an abortive entry into the mitotic prophase stimulated by the transient activation of the cyclin B-dependent kinase-1 (Cdk1; 17), accompanied by the permeabilization of the nuclear envelope, the nuclear translocation of mammalian target of rapamycin (mTOR), the mTOR-mediated phosphorylation of p53 on serine 15 (p53S15P; 18), the p53-

generated by coculture of HeLa cells expressing HIV-1 Env and HeLa cells expressing CD4. Representative examples of p53S15P+ p53S46P- (E) and p53S15P+ p53S46P+ (F) cells, 36 h after beginning of coculture, are shown. No p53S15P- p53S46P+ were detectable and all syncytia positive for p53S15P or p53S46P exhibited karyogamy (nuclear fusion), as detectable by staining with Hoechst 33342. (G) Quantitation of the frequency of syncytia with positive and negative staining for p53S15P or p53S46P, as determined in C and D. Results are means of three experiments \pm SD. (H) Effect of different inhibitors on p53S15P and p53S46P in Env-elicited syncytia. During coculture (36 h), HeLa Env and HeLaCD4 cells were incubated in the presence of the Cdk1 inhibitor roscovitine, the mTOR inhibitor rapamycin, the p53 inhibitor cyclic α -pifithrin, and the pan-caspase inhibitor Z-VAD.fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone), followed by analysis of karyogamy, p53S15P, p53S46P, and nuclear apoptosis. Results are means \pm SD of three independent experiments. Asterisks indicate significant ($P < 0.01$, paired Student's *t* test) inhibitory effects.

mediated transcription of proapoptotic proteins including Puma (19) and Bax (18), Puma-dependent insertion of Bax into mitochondrial membranes (19), and finally Bax-mediated mitochondrial release of cytochrome *c* with subsequent caspase activation (20).

Several observations suggest that p53 acts as an essential transcription factor in the apoptotic process elicited by HIV-1 Env. First, the activating phosphorylation of p53 on serine 15

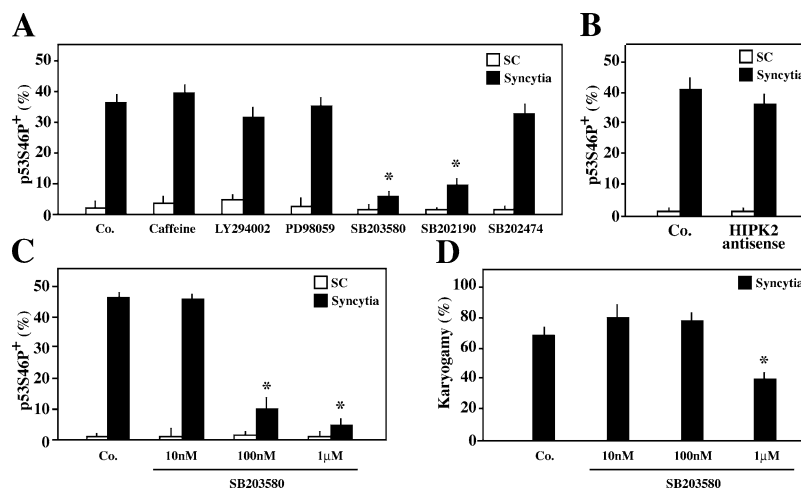


Figure 2. Identification of p38 MAPK as a putative p53 S46-phosphorylating enzyme. (A) Screening of putative p53-phosphorylating kinases. HeLa Env/CD4 cocultures (36 h) were performed in the presence of the indicated inhibitors (500 μ M caffeine, 5 μ M PD98059, and 100 nM of the SB series), and the frequency of p53S46P⁺ syncytia or single cells (SC) was determined by immunofluorescence as in Fig. 1 C. (B) Effect of HIPK2-antisense oligonucleotide on p53S46P. HeLa Env/CD4 were transfected with a HIPK2-antisense oligonucleotide or a scrambled control oligonu-

cleotide for 24 h before initiation of coculture and then cocultured for 36 h, followed by scoring of p53S46P⁺ syncytia. (C and D) Dose response of the inhibitory effect of SB203580. HeLa Env/CD4 cocultures (36 h) were performed in the presence of variable concentrations of SB203580, and the frequency of p53S46P⁺ (C) and karyogamic syncytia (D) was determined. Results are means \pm SD of three determinations, and asterisks indicate significant ($P < 0.01$) effects.

is found in lymphocyte (21) or monocyte (17) cultures infected with HIV-1 in vitro, in lymph node biopsies from HIV-1-infected donors (18), as well as peripheral blood mononuclear cells of HIV-1-infected individuals, correlating with viral load (17). p53 was also found to accumulate in the cortex of patients with HIV-associated dementia (22, 23). Second, transfection with dominant-negative (DN) p53 mutants or treatment with a pharmacological p53 inhibitor, cyclic pifithrin- α (24), prevents the Env-induced up-regulation of Bax and thus retards syncytial cell death in vitro (17, 18). Similarly, neurons and microglia cells from p53^{-/-} mice are resistant against the lethal effect of recombinant gp120 (23). Third, transcriptome analyses performed on HIV-1-infected cultures revealed the induction of p53 target genes including Bax (21, 25), and the p53-target gene Puma was found to be up-regulated in lymph nodes and peripheral blood mononuclear cells from HIV-infected individuals (19).

The activation of the mitochondrial death pathway by p53 involves transcriptional (26) and perhaps nontranscriptional effects (27). The transcriptional activity of p53 and its preferential activation on apoptosis-inducing (rather than cell cycle-arresting) genes depends on a series of posttranscriptional modifications, one of which is phosphorylation of p53 on serine 46 (p53S46P; 28, 29). This activating phosphorylation can be mediated by ataxia telangiectasia-mutated protein (presumably in an indirect fashion; 30) and directly by homeodomain-interacting protein (HIP) kinase-2 (31, 32), and perhaps p38 MAPK (33, 34), although this latter interaction has not yet been shown to be direct. To further characterize the role of p53 in HIV-1 Env-induced apoptosis, we therefore decided to investigate the implication of p53S46P and putative p53S46P kinases in the death pro-

cess. Here, we describe that p53S46P mediated by p38 MAPK is a critical event of Env-induced apoptosis.

RESULTS

Phosphorylation of p53 on serine 46 (p53S46P) elicited by HIV-1 Env and HIV-1 infection in vitro and in vivo

Lymph node biopsies from several untreated HIV-1-infected individuals were found to contain a substantial fraction of cells whose nucleus stained positively with a phosphoepitope-specific antibody recognizing p53S46P. Frequently, such p53S46P⁺ cells contained several nuclei and thus were bona fide syncytia. In contrast, no such p53S46P⁺ staining was found in biopsies from healthy controls (Fig. 1 A). p53S46P⁺ cells were also found among circulating PBMC from HIV-1-infected patients, yet disappeared after successful antiretroviral therapy (Fig. 1 B). Among untreated patients, a positive correlation between the frequency of p53S46P⁺ PBMC and viral titers was found (Fig. 1 C). Multinuclear cells expressing p53S46P were also observed in postmortem brain sections from patients with HIV-1-associated dementia, in 17 out of 17 cases (Fig. 1 D). Such p53S46P-expressing multinuclear cells stained positively for the HIV-1 antigen p24 (unpublished data), suggesting that they result from direct HIV-1 infection (35). Multinuclear cells were absent in HIV-1-infected patients without neurological symptoms ($n = 10$), as well as in uninfected controls (36). Moreover, syncytia formed by coculture of Env-transfected HeLa cells with a CD4-CXCR4-expressing HeLa cell line exhibited p53S46P, as detectable by two different methods, namely immunofluorescence staining (Fig. 1, E-G) and immunoblotting (see Fig. 3 D and Fig. 9 B). Double staining experiments revealed that all p53S46P⁺ syncytia

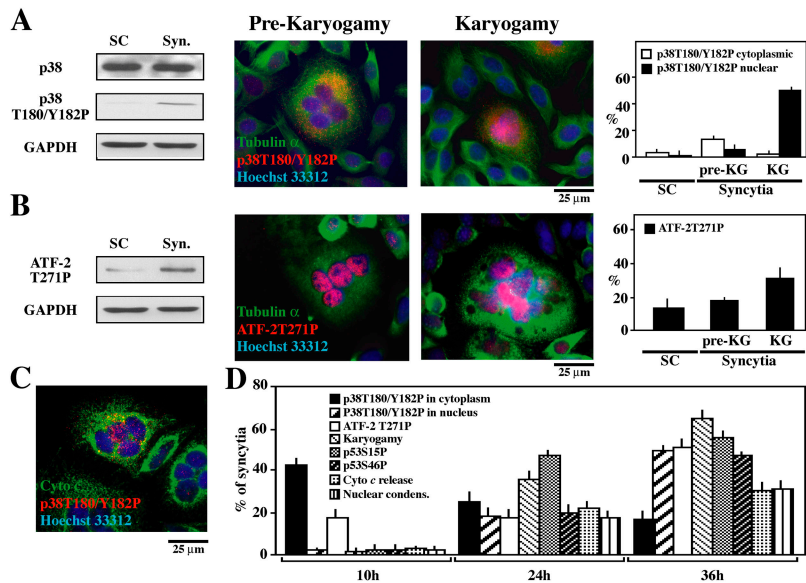


Figure 3. Phosphorylation of p38 MAPK and ATF-2 in Env-elicited syncytia. (A and B) Detection and subcellular localization of p38 MAPK phosphorylated on T180/Y182 (p38T180/Y182P) and ATF-2 phosphorylated on T271 (ATF-2T271P). HeLa Env/CD4 cocultures (Syn., 36 h) or freshly mixed single cell (SC) suspensions were subjected to immunoblot detection of p38 (irrespective of its phosphorylation status), p38T180/Y182P (A), or ATF-2T271P (B). Moreover, immunofluorescence stainings for the detection of p38T180/Y182P (A) or ATF-2T271P (B) were performed and representative examples of prekaryogamic (pre-KG) and karyogamic (KG)

syncytia are depicted. Note that p38T180/Y182P is cytoplasmic in prekaryogamic syncytia and nuclear in karyogamic syncytia. (C and D) Temporary relationship of p38T180/Y182P with regard to apoptosis. Double staining experiments revealed that nuclear p38T180/Y182P was detectable whereas cytochrome *c* was still retained in a punctate cytoplasmic (mitochondrial) localization (C). Kinetic analyses indicate that nuclear p38T180/Y182P accumulation correlates with p53S46P (D) yet occurred before cytochrome *c* release and nuclear chromatin condensation.

were p53S15P⁺ (Fig. 1 F), whereas some p53S15P⁺ syncytia were p53S46P⁻ (Fig. 1 E), and very few if any p53S15P⁻ p53S46P⁺ (Fig. 1 G). This suggests that p53 is first phosphorylated on S15 and then on S46, as corroborated by kinetic studies (see Fig. 3 D). Inhibition of Cdk1 (with roscovitine) and mTOR (with rapamycin), which both inhibit karyogamy (17), both reduced p53S15P and p53S46P. In contrast, inhibition of p53-mediated transactivation (with cyclic pifithrin- α) and apoptotic caspase activation (with Z-VAD.fmk [N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone]) had no effect on p53S15P and p53S46P (Fig. 1 H). As an internal control of their efficacy, all these treatments significantly reduced syncytial apoptosis. Altogether, these findings suggest that p53S15P and p53S46P occur as functionally linked, yet sequential events, independently from p53-mediated transcriptional effects and upstream from apoptosis.

Identification of the p53S46 kinase stimulated by HIV-1 Env as p38 MAPK

Inhibition of putative p53S46 kinases with caffeine (which inhibits ataxia telangiectasia-mutated protein) (37), LY294002 (which inhibits PI3 kinases; 38), PD98059 (which inhibits ERK1/2; 39), or transfection with an antisense oligonucleotide designed to down-regulate the expression of HIP kinase-2 (31), had no effect on the frequency of p53S46P⁺ syncytia (Fig. 2, A and B) and did not reduce the propensity of syncytia to undergo apoptosis (unpublished data). In strict

contrast, SB203580, an inhibitor of p38 MAPK (40), strongly reduced p53S46P. The inhibition of p53S46P was also observed with another p38 MAPK inhibitor, SB202190 (41), but not with the inactive analogue SB202474 (Fig. 2 A). The inhibition of p53S46P with SB203580 was near-to-optimal at 100 nM (Fig. 2 C), that is at a concentration reported to be specific for p38 MAPK (40). At this dose, SB203580 had no effect on syncytium formation (unpublished data) and karyogamy (Fig. 2 D). Using an antibody that recognizes phosphorylated p38 MAPK (p38T180P-Y182P), we detected activate p38 in HeLaEnv-CD4 cocultures. At the prekaryogamic stage, p38T180P-Y182P was confined to the cytoplasm. During karyogamy, however, p38T180P-Y182P concentrated in the nucleus (Fig. 3 A). The relocalization of p38T180P-Y182P correlated with the phosphorylation of one of its nuclear targets, ATF-2 (42), on T271 (ATF-2 with phosphorylated tyrosine 271 [ATF-2T271P]; Fig. 3 B), suggesting that nuclear p38 MAPK is truly active. Syncytia became p38T180P-Y182P⁺ (Fig. 3 C) and ATF-2T271P⁺ (unpublished data) before their mitochondria released cytochrome *c* and their nuclei condensed, as confirmed by kinetic analyses (Fig. 3 D). Thus, p38 MAPK activation occurs before apoptosis. Importantly, p38T180P-Y182P was also detected in HIV-1-infected cultures, shortly upon productive infection (Fig. 4 A), in a fraction of lymph node cells from untreated HIV-1-infected donors with high viral titers (Fig. 4 B), in PBMC from untreated HIV-1 carriers, correlating with viral load (Fig. 4 C), as well as in syncy-

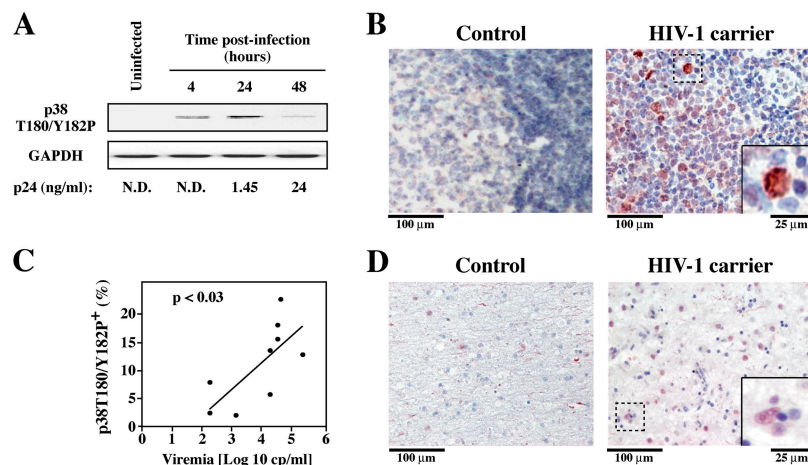


Figure 4. Detection of activated p38 MAPK (p38T180/Y182P) in HIV-1 infection. (A) In vitro induction of p38T180/Y182P. C8166 cells were infected with a clinical HIV-1 isolate as detailed in the Materials and Methods section. The amount of p24 was quantified by ELISA in the supernatants of cultures. (B) Presence of p38T180/Y182P in lymph node biopsies from a freshly diagnosed, untreated HIV-1 carrier with viral loads $>10^5$ copies/ml. $83 \pm 8\%$ ($x \pm SD$, $n = 6$) of the lymph node cells from such HIV-1 carriers were scored as positive for p38T180/Y182P, whereas control biopsies from noninfected individuals lack p38T180/Y182P ($<1\%$ positive). (C) Correlation between the frequency of p38T180/Y182P⁺ PBMC and viral titers in untreated HIV-1 carriers. (D) Detection of p38T180/Y182P in cortical brain sections of individuals with clinically manifest HIV-1-associated dementia. The insert shows a multinucleated p38T180/Y182P⁺ cell. Such images have been obtained in 17 different individuals with HIV-1-associated dementia but not in 10 different control subjects. Among multinucleated cells, $63 \pm 19\%$ ($x \pm SD$, $n = 9$) were found to express p38T180/Y182P.

Selective inhibition of p53S46P abolished p53-mediated transcription and apoptosis in Env-elicited syncytia

As shown above, specific inhibition of p38 MAPK by SB203580, used at a concentration of 100 nM, fully prevents the apoptogenic phosphorylation of p53 on S46 in HeLa Env-CD4 syncytia. At this dose, however, SB203580 had no effect on the syncytial accumulation of cyclin B (unpublished data) and thus did not prevent karyogamy (Fig. 6 A). Very similar results, that is inhibition of ATF-2T271P and p53S46P (but not p53S15P), were obtained when p38 MAPK was neutralized by transfection with a DN p38 mutant (43) (Fig. 6 B) or when p38 MAPK was knocked down with a p38-specific small interfering RNA (siRNA; Fig. 6, C and D). Although p38 suppression by itself did not inhibit p53S15P (Fig. 6, A–C), the combined inhibition of p38 (with SB203580) and another established p53S15 kinase, mTOR (with rapamycin), had a synergistic inhibitory effect (Fig. 6 E), suggesting that p38 MAPK has some

cells, $n = 5$). The insert shows a putative syncytium. (C) Correlation between the frequency of p38T180/Y182P⁺ PBMC and viral titers in untreated HIV-1 carriers. (D) Detection of p38T180/Y182P in cortical brain sections of individuals with clinically manifest HIV-1-associated dementia. The insert shows a multinucleated p38T180/Y182P⁺ cell. Such images have been obtained in 17 different individuals with HIV-1-associated dementia but not in 10 different control subjects. Among multinucleated cells, $63 \pm 19\%$ ($x \pm SD$, $n = 9$) were found to express p38T180/Y182P.

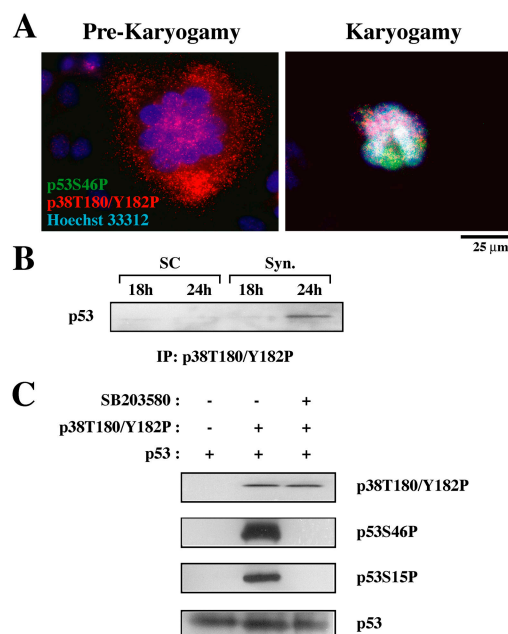


Figure 5. Direct phosphorylation of p53 on S46 by p38 MAPK. (A) Coimmunolocalization of p38T180/Y182P and p53S46P. HeLa Env/CD4 cocultures (36 h) were subjected to simultaneous detection of p38T180/Y182P and p53S46P with monoclonal mouse and polyclonal rabbit antibodies, revealed by red and green fluorescence, respectively. Note the significant coimmunolocalization (yellow). (B) Coimmunoprecipitation of p38T180/Y182P and p53. Extracts from HeLa Env/CD4 cocultures were subjected to immunoprecipitation (IP) of p38T180/Y182P, followed by immunodetection of p53 in the immunoprecipitate. (C) Direct phosphorylation of p53 by p38 MAPK in vitro. Recombinant p38 and p53 were mixed together in the presence of 1 mM ATP and in the absence or presence of the p38 MAPK inhibitor SB203580 (100 nM), followed by immunodetection of p53 (phosphorylated or unphosphorylated), p53S15P, and p53S46P. These experiments were repeated twice, yielding similar results.

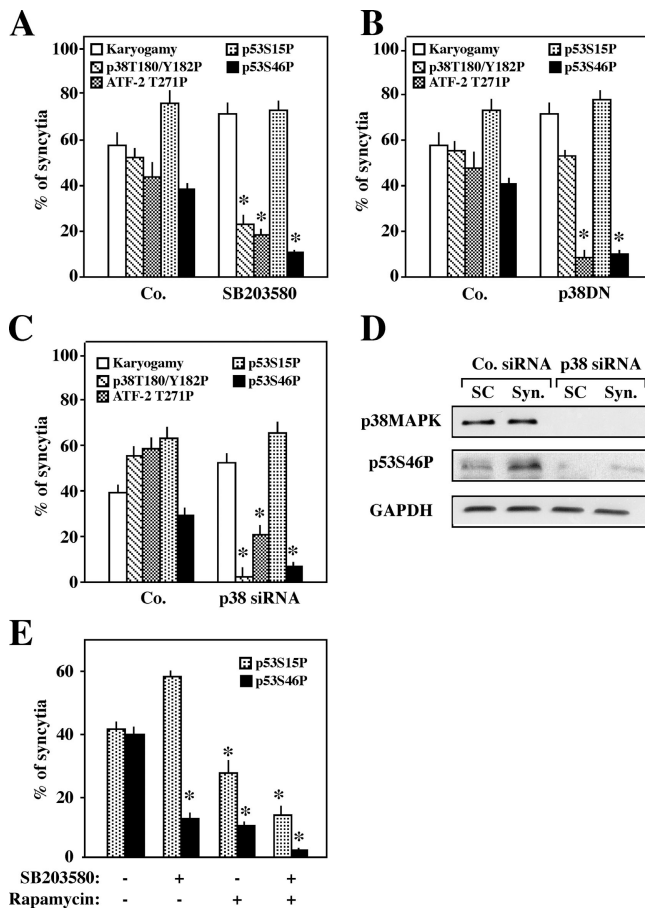


Figure 6. Specific requirement of p38 MAPK for p53 phosphorylation on S46 in Env-elicited syncytia. (A–C) Effect of p38 MAPK kinase inhibition of phosphorylation events detected by immunofluorescence. Three different methods of p38MAPK kinase inhibition were employed: addition of a chemical inhibitor (100 nM SB203580) from the beginning of coculture lasting 36 h (A), transfection with a DN p38 mutant 24 h before initiation of coculture (B, control/transfection with vector only), and p38-specific siRNA 24 h before coculture (C, control/irrelevant siRNA). Results are means of three independent experiments ($x \pm SD$, $n = 3$) and asterisks indicate significant ($P < 0.01$) differences with respect to controls. (D) Confirmation of the effect of p38 siRNA by immunoblot. Syncytia obtained by cocultures as in C were subjected to immunoblot detection of p38 MAPK, p53S46P, and GAPDH. (E) Effect of the combined inhibition of p38 MAPK and mTOR on p53S15P. Cells were cultured in the presence of SB203580 (100 nM) and/or rapamycin (1 μ M) for 30 h, and the number of p53S15P⁺ cells was scored by immunofluorescence. Asterisks indicate significant ($P < 0.01$) inhibitory effects.

p53S15 kinase activity in vivo, in accord with the in vitro data (Fig. 5 C).

Next, we transfected HeLa Env and HeLa CD4 cells with a p53-inducible green fluorescent plasmid (GFP) reporter gene, followed by coculture of the two cell lines to generate syncytia, which express GFP (whereas single cells remain GFP negative). The two p38 MAPK inhibitors (SB203580 or SB202190) and DN p38 strongly inhibited the syncytial expression of p53-inducible GFP (Fig. 7 A) and of a p53-inducible luciferase reporter construct (Fig. 7 B), respectively. Thus, the selective inhibition of p53S46P

(without any effect on p53S15P) suffices to block the transactivating function of p53, at least in this system. Because apoptosis of Env-elicited syncytia depends on p53-mediated transcriptional effects (as described in the introduction), p53S46P inhibition should result in the interruption of the lethal signal transduction cascade. Accordingly, SB203580 and DN p38 inhibited the conformational activation of Bax and Bak that normally occurs in untreated syncytia (detectable by immunofluorescence with antibodies recognizing the exposed NH₂ termini of Bax or Bak), the release of cytochrome *c* from mitochondria (detectable by immunostaining) and apoptotic nuclear condensation (Fig. 7, C and D). p38 inhibition also abolished the p53-dependent induction of the proapoptotic Bax/Bak activator Puma, whereas inhibiting syncytial apoptosis (Fig. 7 E). Altogether, these data underscore the critical apoptogenic role of p38 MAPK-mediated p53S46P for Env-induced apoptosis.

Upstream signaling events leading to p38 MAPK activation

In a further series of experiments, we addressed the question as to whether cell fusion per se would be sufficient to elicit p38 activation and apoptosis or whether Env-mediated signal received via CD4 and CXCR4 would be required for this process. HeLa CD4 and HeLa Env cells fused with polyethylene glycol (PEG) still exhibited a significant level of p38 MAPK phosphorylation when C34 (an inhibitor of the interaction of Env with the CD4–CXCR4 complex) was added into the system. Similarly, PEG-enforced homotopic fusion of HeLa CD4 cells or HeLa Env cells resulted in p38 activation, p53S46P and apoptosis (Fig. 8). Thus, heterotopic interactions between Env and CD4–CXCR4 are not absolutely required for the activation of p38. Nonetheless, the interaction between Env and CD4–CXCR4 yields a higher level of p38 activation than when it is blocked, either by the C34 peptide or by the elimination of either CD4 or Env from the system (Fig. 8). In a further series of experiments, Env-elicited syncytia were found to exhibit an activating phosphorylation of the MAPK kinase MKK3 or 6 on S189 or S207, respectively, using a bispecific antibody (which recognizes phosphorylated forms of either MKK 3 and 6; Fig. 9 A). Transfection with DN mutants of MKK3 or MKK6 inhibited the appearance of p38T180P–Y182P and p53S56P in Env-elicited syncytia (Fig. 9 B). Moreover, DN MKK3 or DN MKK6 abolished the transcriptional activity of p53 in this system (Fig. 9 C) and suppressed all mitochondrial and nuclear manifestation of apoptosis, yet had no effect on karyogamy and p53S15P (Fig. 9 D). Thus, MKK3/6 is indispensable for p38 MAPK activation, p53 phosphorylation on S46 (but not S15), and p53-dependent apoptosis. Altogether, these data point to a cascade in which syncytium formation results in the activation of the lethal MAP kinase cascade.

DISCUSSION

The data shown in this paper establish that p38 MAPK can phosphorylate p53S46P, thereby triggering the proapoptotic

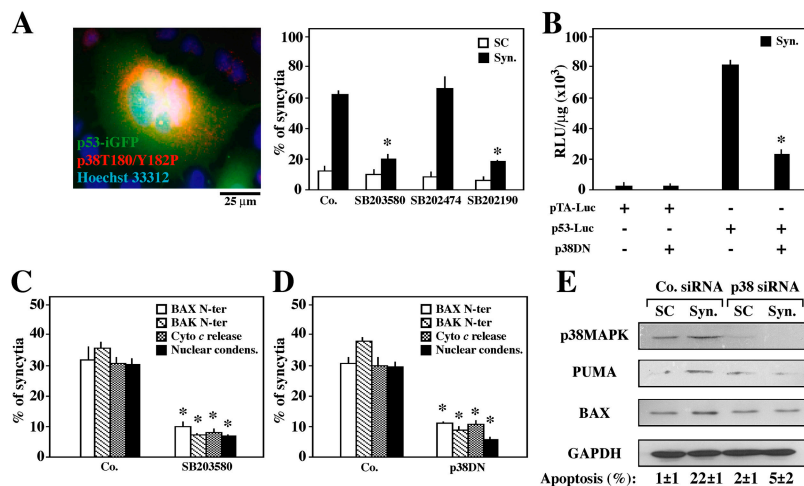


Figure 7. Abolition of p53-mediated transcription and apoptosis induction by inhibition of p38 MAPK. (A) Effect of chemical p38 MAPK inhibitors on Env-induced p53-mediated transcription within syncytia. A p53-inducible GFP reporter gene was transfected into HeLa Env/CD4 cells 24 h before coculture for 36 h, resulting in expression of GFP mainly within syncytia, but not single cells (left panel). The effect of the p38 MAPK inhibitors SB203580 and SB202190 (as well as that of the ineffective control compound SB202474) is shown in the right panel. (B) Effect of DN p38. HeLa Env/CD4 cells were transfected with a p53-inducible luciferase reporter construct (p53-Luc), a noninducible luciferase construct (pTA-Luc), with p38 DN or its empty vector, 24 h before coculture, followed by cocultivation of HeLa Env and HeLa CD4 cells for 36 h and determination of luciferase

activity. (C and D) Effect of pharmacological p38 MAPK inhibitor (C) or DN p38 (D) on the immunofluorescence-detectable exposure of the Bax or Bak NH₂ termini, the mitochondrial release of cytochrome c, and Hoechst 33342-detectable chromatin condensation 36 h after syncytium formation. Note that these parameters are largely negative in single cells (<5%). (E) Effect of p38 MAPK knock-down by siRNA. Cells were subjected to siRNA 24 h before coculture for 36 h (syncytia, Syn) or separate coculture for 36 h (single cells, SC), and the expression of p38 MAPK, PUMA, and Bax was monitored by immunoblot. The frequency of apoptotic cells was determined among single cells and syncytia by Hoechst 33312 staining and fluorescence microscopy.

transactivating function of p53. This notion is based on the strong correlation between the activatory p38 phosphorylation (p38T180P–Y182P) and p53 phosphorylation on S46 (p53S46P), in Env-elicited syncytia generated in vitro (Figs. 1 and 3), in HIV-1-infected cultures of CD4⁺ cells (Fig. 4 A), as well as in lymph nodes, PBMC, and cortical sections from HIV-1-infected individuals (Fig. 1, A–D and Fig. 4, B–D). Inhibition of p38 MAPK by a variety of different techniques (pharmacological agents, siRNA, transfection with DN mutants of either p38 MAPK or MKK3/6) prevented p53S46P and blocks apoptosis, thus establishing the cause and effect relationship between p38 MAPK activation,

p53S46P, and subsequent cellular demise (Figs. 2 and 5–8). It appears plausible that p38 phosphorylates p53 in a direct fashion because both proteins colocalized and coimmunoprecipitated in Env-elicited syncytia (Fig. 5, A and B) and because recombinant p38 MAPK phosphorylated recombinant p53 on S46 in vitro (Fig. 5 C). As a side observation, p38 MAPK also phosphorylated p53 on S15 (Fig. 5 C), but this reaction is likely to play a minor role in vivo, given that p38 MAPK inhibition alone did not reduce p53S15P and only became effective when the principal p53S15 kinase operating in this system, mTOR (17, 18), was also inhibited (Fig. 6 E).

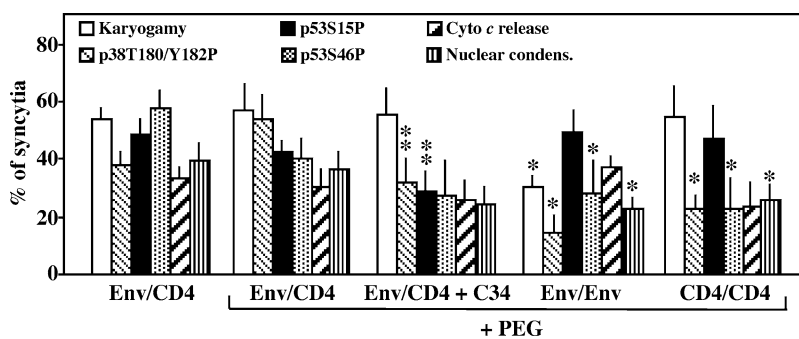


Figure 8. Cell fusion for activation for p38 MAPK cascade. HeLa Env and HeLa CD4 cells were fused in the indicated heterotopic or homotopic combination with polyethyleneglycol, followed by coculture for 36 h in the presence or absence of C34 (which inhibits the interaction between CD4 and Env). Then, the frequency of syncytia exhibiting activating phosphorylation of p38, p53, and mitochondrial or nuclear characteristics of

apoptosis was determined by immunofluorescence staining. Double asterisks indicate significant effects of C34 as compared with HeLa Env plus HeLa CD4 cells fused with PEG. Single asterisks show significant ($P < 0.01$, Student's *t* test) differences between the PEG-enforced fusion of HeLa CD4 or HeLa Env cells, as compared with the spontaneous fusion between HeLa Env and HeLa CD4 cells.

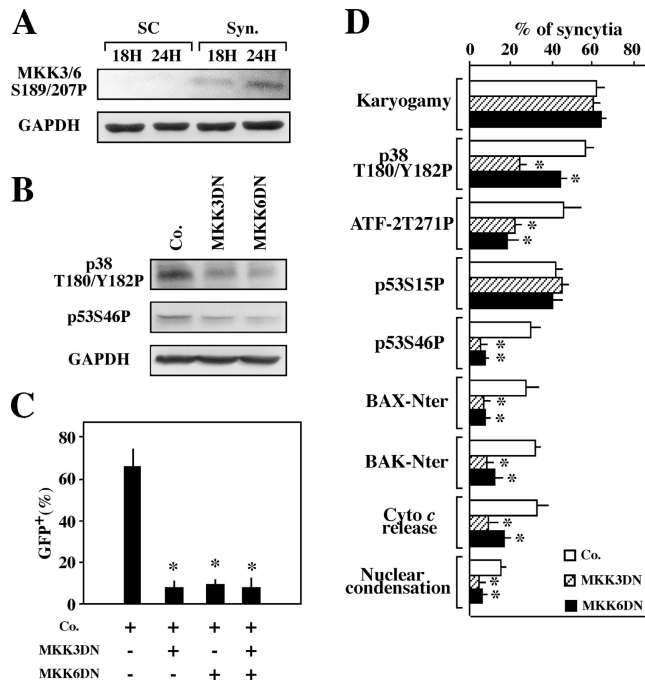


Figure 9. Activation of MKK3/6 upstream of p38 MAPK in Env-elicited syncytia. (A) Detection of the activated, phosphorylated form of MKK3/6 (MKK3/6 S89/207P) in separate or cocultures of HeLa Env and HeLa CD4 cells. (B) Effect of DN MKK3 and MKK6 on the phosphorylation of p38 and p53. HeLa Env/CD4 cells were transfected with the indicated construct (or vector only; control [Co.]), 24 h before coculture for 36 h, and the presence of the indicated antigens was determined. (C) Effect of DN MKK3 and MKK6 on p53-mediated transactivation. Cells were simulta-

An interesting problem arises from the fact that p38 MAPK activation is a frequent stress response, which normally does not lead to cell death (44–46). One mechanism of regulation could be the intracellular localization of p38 MAPK. Whereas p38T180P–Y182P is confined to the cytoplasm, early after syncytial formation, it later moves to the nucleus, when the nuclear envelope loses its barrier function due to the action of the mitotic Cdk1–cyclin B complex (Fig. 3 A). Hence, inhibition of Cdk1–cyclin B prevents the nuclear relocalization of p38T180P–Y182P (unpublished data) and concomitantly prevents the phosphorylation of nuclear p38 MAPK targets such as ATF-2 and p53 (Fig. 1 G). Thus, a combination of aberrant cell cycle regulation and kinase activation may have novel effects when p38 MAPK gains access to the nucleus and hence to substrates from which it usually is separated. In this context, it is intriguing that p38 MAPK can actually mediate a G2/M arrest in response to different cellular stressors including DNA damage (47, 48) and osmotic stress (49), presumably through inactivation of the cell cycle regulator CDC25C phosphatase and its downstream target Cdk1. However, in the paradigm of syncytial apoptosis studied here, inhibition of p38 MAPK did not release the cells from G2/M arrest and thus did not provoke an increase in syncytial divisions.

It is important to note that p38 MAPK is a pleiotropic kinase that, in the context of HIV-1 infection, is not only in-

neously transfected with p53-inducible GFP, in combination with empty vector, DN MKK3 or DN MKK6, 24 h before coculture and the frequency of GFP-positive syncytia was scored after 36 h of coculture. (D) Effect of DN MKK3 and MKK6 on the apoptotic cascade elicited by Env. The indicated phenomena were detected by immunofluorescence, in three independent experiments. Results are means \pm SD ($n = 3$), and asterisks indicate significant ($P < 0.01$) inhibitory effects, as compared with vector-only transfected controls.

involved in the apoptotic cascade triggered by syncytium formation. It has been shown that pharmacological inhibition of p38 can actually reduce HIV-1 replication in vitro, via an unknown mechanism (50, 51). The data contained in this paper suggest that, in addition, p38 may be cardinal for the transmission of lethal signals elicited by Env, even in the absence of a virus, that is in the context of bystander killing. Thus, p38 might become an attractive (therapeutic or experimental) target, either for suppressing HIV-1 replication or for reducing deleterious host responses to viral products such as Env.

MATERIALS AND METHODS

Cells and culture conditions. HeLa cells stably transfected with the *Env* gene of HIV-1^{LAI/IIIIB} (HeLa Env) and HeLa cells transfected with CD4 (HeLa CD4) were cocultured at a 1:1 ratio, in Dubelcco's modified Eagle's medium supplemented with 10% FCS, L-glutamine, in the absence or presence of 1 μ M roscovitine (Calbiochem-Novabiochem), 1 μ M rapamycin (Sigma-Aldrich), 10 μ M cyclic pifithrin- α , 100 μ M ZVAD (Bachem), 500 μ M caffeine (Calbiochem-Novabiochem), 1 μ M LY294002 (Sigma-Aldrich), 5 μ M PD98059 (Calbiochem-Novabiochem), p38 MAPK inhibitors (SB203580 and SB202190), and an inactive analogue of p38 MAPK inhibitors, SB202474 (Calbiochem-Novabiochem). HeLa CD4 or/and HeLa Env were also cultured on coverslips and were fused with PEG, as described previously (17–20).

Plasmids, transfection, and p53-mediated transcription activity. HeLa CD4 and HeLa Env cells were transfected with the p53 luciferase reporter construct p53-Luc alone (Mercury pathway profiling system from CLONTECH Laboratories, Inc.; 1 μ g DNA) or together with the control

luciferase reporter construct or with the p38 dominant negative mutant (p38DN; reference 43; 1 μ g DNA). Transfections were performed using 2 μ l Lipofectamine 2000 (Invitrogen) 24 h before fusion. Luciferase activity was measured after 24 h of coculture using the luciferase reporter assay kit from CLONTECH Laboratories, Inc. Transfections of a p53-responsive enhanced GFP (19; gift from Klas Wiman, Karolinska Hospital, Stockholm, Sweden) with pcDNA3.1 vector alone (control) (1 μ g DNA) or with a DN MKK3 mutant (pcMKK3DN S189A/T193L) or/and with MKK6 DN plasmids (pcMKK6DN S207A/T211L; reference 52; 1 μ g DNA) were performed 24 h before coculture of HeLa CD4 and HeLa Env cells, using transfection's protocol described above. The frequency of GFP-expressing cells was assessed by immunofluorescence.

In vitro kinase assays. Kinase reactions were performed in 50 μ l of kinase buffer (50 mM Hepes, pH 7.4, 25 mM MgCl₂, 25 mM β -glycerophosphate, 2 mM DTT and 0.1 mM Na₃VO₄) containing 1 μ g of an active GST-p38 recombinant protein (Chemicon), 1 mM ATP and 2 μ g of fusion protein corresponding to the full-length p53 protein of human origin as substrate (Santa Cruz Biotechnology, Inc.). Reactions were performed for 30 min at 30°C and stopped by adding 50 μ l of 2 \times sample buffer and boiling for 5 min. Samples were resolved by SDS-PAGE. The phosphorylation status of p53 was monitored by immunoblotting with p53S15P and p53S46P antibodies.

Immunoblot and immunoprecipitation. Total cellular proteins were extracted in lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 10 mM Na₃VO₄, 10 mM NaF, 5 mM DTT, 3 mM Na₄P₂O₇, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin). Aliquots of protein extracts (30 μ g) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. After incubation for 12 h with membrane blocking solution (Zymed Laboratories), membranes were incubated with antibodies specific for p38, p38T180/Y182P [p38 with phosphorylated threonine 180 and tyrosine 182], ATF-2T271P, p53S15P, p53S46P, and p53 (Cell Signaling Technology), Puma (US Biological), or GAPDH (mAb from Chemicon). For immunoprecipitation, lysates (200 μ g) were incubated overnight at 4°C with affinity-purified rabbit polyclonal antibodies specific for p38T180–Y182P. Immunocomplexes were recovered by the addition of protein A/G-agarose beads (1 h at 4°C; Santa Cruz Biotechnology, Inc.), washed six times with lysis buffer, and subjected to SDS-PAGE. Immunoblot analysis was performed with a p53-specific mAb (Santa Cruz Biotechnology, Inc.).

Immunofluorescence. Cocultures were fixed with 4% paraformaldehyde/0.19% picric acid/phosphate-buffered saline for 30 min, permeabilized with 0.1% SDS for 10 min, and incubated with FCS for 20 min. Rabbit antisera specific for p53S15P, p53S46P, p38T180/Y182P, ATF-2T271P (Cell Signaling Technology), or the NH₂ terminus of Bax (N20; Santa Cruz Biotechnology, Inc.) were used for immunodetection in PBS containing 1 mg/ml BSA and revealed with goat anti-rabbit IgG conjugated to Alexa 568 (red) or Alexa 488 (green) fluorochromes from Molecular Probes. Cells were also stained for the detection of the NH₂ terminus of Bak (Ab1; Oncogene Research Products), p53S15P (mAb), p38T180/Y182P (mAb; Cell Signaling), revealed by anti-mouse IgG Alexa conjugates. To assess karyogamy and apoptotic chromatin condensation, cells were counterstained with Hoechst 33342 (Molecular Probes).

Antisense constructs and RNA interference. The inhibition of endogenous HIPK2 expression was performed with HPLC-purified antisense phosphorothioate oligonucleotides (HIPK2 antisense: 5'-CGCGGTTTCATGGCAACGGAGAAGGG-3'; control: 5'-CCTCTTACCTCAGTTCAATTTATA-3'; GeneSet Oligos, Paris, France; 32). The HIPK2 or control antisense phosphorothioate oligonucleotides (100 nM) were transfected with 2 μ l of Oligofectamine Reagent (Invitrogen) into HeLa CD4 and HeLa Env, 24 h before coculture. RNA interference of p38MAPK expression was obtained using the p38 siRNA/siAB Assay Kit (Upstate Biotechnology), following the manufacturer's protocol. Both HeLa CD4 and

HeLa Env cells were transfected with a pool of four selected specific p38MAPK α siRNA duplexes or with nonspecific siRNA duplexes, 24 h before coculture.

Patients' samples and in vitro infection with HIV-1 isolate. Axillary lymph node biopsies were obtained from healthy and HIV-1-infected individuals (all males, mean age 36 yr, with a plasma viral load >10⁵ copies/ml) after informed consent. Plasma HIV-1 RNA levels were determined by the bDNA procedure (Versant HIV RNA 3.0) according to the manufacturer's instructions (Bayer). None among the HIV-1⁺ patients received interferons, glucocorticoids, was positive for hepatitis B or C, or exhibited signs of autoimmunity. Postmortem frontal cortex sections were obtained from 17 brains of patients with HIV-1-associated dementia (but lacking secondary infections), 10 HIV-infected patients without neurological symptoms, and 3 control brains obtained from uninfected control patients. These autopsy samples from patients enrolled in a prospective studies on HIV-related dementia were obtained from the Institute of Pathological Anatomy, Catholic University of Rome, School of Medicine (Rome, Italy). Biopsies and postmortem samples were obtained in accord with the national legislation, after approval by the Institutional Review Board of the National Institute for Infectious Disease "Lazzaro Spalanzani." After fixation with 10% formalin and dehydration, frontal cortex samples and lymph nodes biopsies were embedded in paraffin. Sections were then cut, deparaffinized, subjected to immunocytochemistry with antibodies specific for p53S46P or p38T180/Y182P (same antibodies as for immunofluorescence), and counterstained with hematoxylin. C8166 cells (10 \times 10⁶) were incubated with a clinical HIV-1 isolates (500 ng of p24) for 4 h at 37°C. After washing out unabsorbed virus, cells (10⁶ cells/ml) were cultured in RPMI medium containing 10% FCS. Total proteins were obtained using TRIzol (Invitrogen) and subjected to immunoblot analysis. PBMC were isolated by Ficoll/Hypaque (Amersham Biosciences) centrifugation of heparinized blood from healthy donors and HIV-seropositive individuals and fixed with 4% formaldehyde in PBS (pH 7.2) before immunohistochemical analyses.

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