Virology 268, 329–344 (2000)
doi:10.1006/viro.1999.0151, available online at http://www.idealibrary.com on IDEAL®

Caspase-Dependent Apoptosis of Cells Expressing the Chemokine Receptor CXCR4 Is Induced by Cell Membrane-Associated Human Immunodeficiency Virus Type 1 Envelope Glycoprotein (gp120)

Martine Biard-Piechaczyk,*[,1] Véronique Robert-Hebmann,* Véronique Richard,† Joachim Roland,* Robert A. Hipskind,† and Christian Devaux*

*Laboratoire Infections Rétrovirales et Signalisation Cellulaire, CNRS EP 2104, Institut de Biologie, 4 Boulevard Henri IV, 34060 Montpellier Cedex, France; and †IGMM, CNRS UMR 5535, IFR 24, Montpellier, France

Received September 7, 1999; returned to author for revision December 8, 1999; accepted December 16, 1999

Human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins interact with CD4 and chemokine receptors on T cells to deliver signals that trigger either activation, anergy, or apoptosis. However, the molecular mechanisms driving these responses remain poorly understood. In this study we demonstrate that apoptosis is induced upon HIV-1 envelope binding to the chemokine receptor CXCR4. Cells expressing a mutant form of CXCR4 with a C-terminal deletion were also sensitive to HIV-1 envelope-mediated apoptosis, indicating that the cytoplasmic tail of CXCR4 is not required to induce the apoptotic pathway. The specificity of this process was analyzed using several inhibitors of gp120-CD4–CXCR4 interaction. Monoclonal antibodies directed against the gp120-binding site on CD4 (ST4) and against CXCR4 (MAB173) prevented the apoptotic signal in a dose-dependent manner. The cell death program was also inhibited by SDF-1α, the natural ligand of CXCR4, and by suramin, a G protein inhibitor that binds with a high affinity to the V3 loop of HIV-1 gp120 envelope protein. These results highlight the role played by gp120-binding on CXCR4 to trigger programmed cell death. Next, we investigated the intracellular signal involved in gp120-induced apoptosis. This cell death program was insensitive to *pertussis toxin* and did not involve activation of the stress- and apoptosis-related MAP kinases $p38^{MAPK}$ and SAPK/JNK but was inhibited by a broad spectrum caspase inhibitor (z-VAD.fmk) and a relatively selective inhibitor of caspase 3 (z-DEVD.fmk). Altogether, our results demonstrate that HIV induces a caspase-dependent apoptotic signaling pathway through CXCR4.

INTRODUCTION

HIV-1 gains entry into susceptible cells through the fusion of viral envelope and cellular membrane. This process is driven by HIV-1 envelope glycoprotein complexes formed by association of $gp120^{env}$ and $gp41^{env}$. For most HIV isolates, fusion is preceded by a high-affinity interaction between HIV-1 $gp120^{env}$ and the extracellular domain 1 of host cell surface CD4, a 58-kDa transmembrane glycoprotein expressed predominantly at the plasma membrane of mature peripheral T helper lymphocytes and macrophages (Arthos *et al.,* 1989; Klatzmann *et al.,* 1984; Littman, 1987; Sattentau *et al.,* 1989). The second step of molecular events leading to fusion is the triggering of conformational changes in the viral spikes (Jones *et al.,* 1998; Lapham *et al.,* 1996; Ugolini *et al.,* 1997). The resulting increased exposure of the $gp120^{env}$ V3 loop allows it to interact with members of the seven-transmembrane (TM)-domain chemokine receptor family, such as CXCR4 and CCR5, which act as coreceptors for T-tropic (X4) and M-tropic (R5) strains, respectively (Alkhatib *et al.,* 1996; Feng *et al.,* 1996). Such interactions are thought to expose the hydrophobic fusion domain of the transmembrane envelope protein $gp41^{env}$, leading to viral entry (Weissenhorn *et al.,* 1997).

The misappropriation of CD4 and chemokine receptor function by HIV-1 likely has important consequences on cell homeostasis and virus propagation. Indeed, both CD4 and chemokine receptors initiate intracellular signaling upon their activation by natural ligands. Stromal cell-derived factor (SDF-1α), the only known natural ligand of CXCR4, activates extracellular signal-regulated kinase (ERK), phosphatidyl-inositol 3′-kinase (PI-3K), and the transcription factor NF-kB and increases phosphorylation of focal adhesion components in cells transfected with CXCR4 (Ganju *et al.,* 1998). During the past few years, a large body of data has indicated that engagement of the cell surface CD4 molecule by HIV-1 $gp120^{env}$ activates signal transduction pathways, such as that involving ERK, which in turn regulates transcription factors like NF-kB, AP1, and Elk1 (Alkhatib *et al.,* 1996; Benkirane *et al.,* 1994, 1995; Briant *et al.,* 1996, 1998; Chirmule *et al.,* 1994; Feng *et al.,* 1996; Popik *et al.,* 1998; Popik and Pitha, 1996; Schmid-Antomarchi *et al.,* 1996).

Since the discovery of seven-TM chemokine receptors as HIV coreceptors, several laboratories have investigated the possibility that HIV triggers intracellular signal-

[1] To whom correspondence should be addressed. Fax: (33)-4-67-60-44-20. E-mail: devaux@sc.univ-montp1.fr.

0042-6822/00 $35.00

ing through these coreceptors. However, only the protein tyrosine kinase Pyk2 was shown to be phosphorylated after HIV-1 envelope binding to CXCR4 HIV-1 coreceptor on T cell lines through a signal transmitted by Gαi proteins (Davis *et al.,* 1997).

In parallel to these activation signals, which probably maximize the production of viral progeny, HIV-1 infection is characterized by a progressive drop of $CD4^+$ T lymphocytes (Fauci, 1993). Both direct and indirect mechanisms of cytopathogenicity have been postulated to cause this cell depletion (Golding *et al.,* 1988; Imberti *et al.,* 1991; Lifson *et al.,* 1986; Sodroski *et al.,* 1986; Somasundaran and Robinson, 1987; Stevenson *et al.,* 1988; Zinkernagel and Hengartner, 1994). HIV-1-induced apoptosis has been proposed as one of the indirect mechanisms (Ameisen and Capron, 1991; Groux *et al.,* 1992; Laurent-Crawford *et al.,* 1991; Martin *et al.,* 1994; Meyaard *et al.,* 1992; Oyaizu *et al.,* 1993; Terai *et al.,* 1991) that act independently of syncytia formation (Connor *et al.,* 1993; Corbeil and Richman, 1995; Maldarelli *et al.,* 1995; Nardelli *et al.,* 1995).

Infectious X4 isolates of HIV-1-induced apoptosis in human T cell lines in which the cytoplasmic tail of CD4 was missing (Guillerm *et al.,* 1998; Jacotot *et al.,* 1997). This suggested that the death program is not triggered by the CD4 molecule but may involve another HIV receptor. Considering the function of CXCR4 in signal transduction, we and others (Guillerm *et al.,* 1998; Jacotot *et al.,* 1997) postulated that the CXCR4 molecule may contribute to HIV-induced apoptosis. Recently, Hesselgesser *et al.* (1998) demonstrated that HIV-1 envelope glycoproteins induce apoptosis through CXCR4 in neuronal cells that do not express CD4. Moreover, Herbein and coworkers (Herbein *et al.,* 1998) reported that apoptosis of $CD8^+$ T cells was triggered through interaction of HIV-1 $gp120^{env}$ with CXCR4 and contacts between membrane-bound tumor-necrosis factor (TNF)-α on macrophages and TNF receptor II on $CD8^+$ cells.

The aim of this study was to analyze the signaling specifically induced through CXCR4 after gp120 contact independently of the biological events triggered via CD4. For this purpose, we constructed a cell model using the HEK cell line cotransfected with CXCR4 and a mutated form of CD4 lacking the cytoplasmic domain which is incapable of activating a signal on its own. We found that HEK cell lines coexpressing this truncated form of CD4 and the CXCR4 molecule, either wild type or truncated after Ala308, were sensitive to HIV-1-induced apoptosis. Our results indicate that while CD4 is necessary for HIV-1 $gp120^{env}$ binding to CXCR4, cell death requires expression of CXCR4 on the target cells. The CXCR4 C-terminal deletion mutant was still active, thus ruling out this domain in apoptotic signaling. Remarkably, apoptosis was dependent on caspases but did not involve Gαi or PI-3K, as it was not inhibited by pertussis toxin or wortmannin. Furthermore $gp120^{env}$ binding to CXCR4 did not induce $p38^{MAPK}$ or stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) activation, which has been coupled to apoptosis in other systems (Paul *et al.,* 1997). Although these observations need to be confirmed in more physiological situations (e.g., CXCR4 positive T cells), it is clearly established in this cellular model that HIV-1 triggers a caspase-dependent apoptosis through CXCR4.

RESULTS

Cellular model

To study the role specifically played by CXCR4 in HIV-1 envelope-induced apoptosis, HEK cells were transfected with the CD4.403 truncated form of CD4, which binds $gp120^{env}$ but is unable to activate a signal on its own, or CD4.403 along with different forms of CXCR4. Clones were selected for surface expression of the transfected gene products and cocultured with 8.E5 cells which express HIV-1 $gp120^{env}$ molecules at their surface. The heterologous HEK cell model system was chosen for several reasons: (i) the parental cell line lacks surface expression of both CD4 and CXCR4 (see below), (ii) this is a cellular model extensively used for the study of G protein-coupled receptor (GPCR)-mediated signals, (iii) this line has been successfully used in many apoptosis studies (Brustugun *et al.,* 1998; Ozawa *et al.,* 1999; Tenneti *et al.,* 1997), and (iv) CXCR4-transfected HEK cells have already proven useful for signaling and HIV-1 coreceptor function studies (Lee *et al.,* 1998; Ling *et al.,* 1999; Wang *et al.,* 1998). It is worth noting that HEK cells do not express the CCR5 gene. Moreover, transfected CXCR4 behaves as the natural molecule in HEK cells, since it supports HIV-1 coreceptor function and transduces signals upon engagement with SDF-1α (see below).

The following stable clones have been constructed for this study: (i) HEK/CD4.403 cells that express a truncated form of CD4 lacking the cytoplasmic domain, (ii) HEK/CXCR4 cells expressing the wild type CXCR4, (iii) HEK/CXCR4.7TM cells that express a truncated form of CXCR4 lacking the last cytoplasmic domain, (iv) HEK/CD4.403/CXCR4 cells expressing the CD4 truncated form CD4.403 and the wild type CXCR4, and (v) HEK/CD4.403/CXCR4.7TM cells expressing the truncated forms of CD4 and CXCR4. Cell surface expression of recombinant molecules was measured by flow cytometry (Fig. 1). Significant expression of CD4.403 and/or CXCR4 was found in the appropriate clones, and the selected clones show nearly identical levels of receptor expression. We note that CD4.403 is expressed at higher levels than CXCR4.

The physiological relevance of our cellular model was tested in several ways. First, we examined the capability of transfected CXCR4 to serve as a coreceptor for entry of an X4 isolate of HIV-1 (Lai strain). Four days after

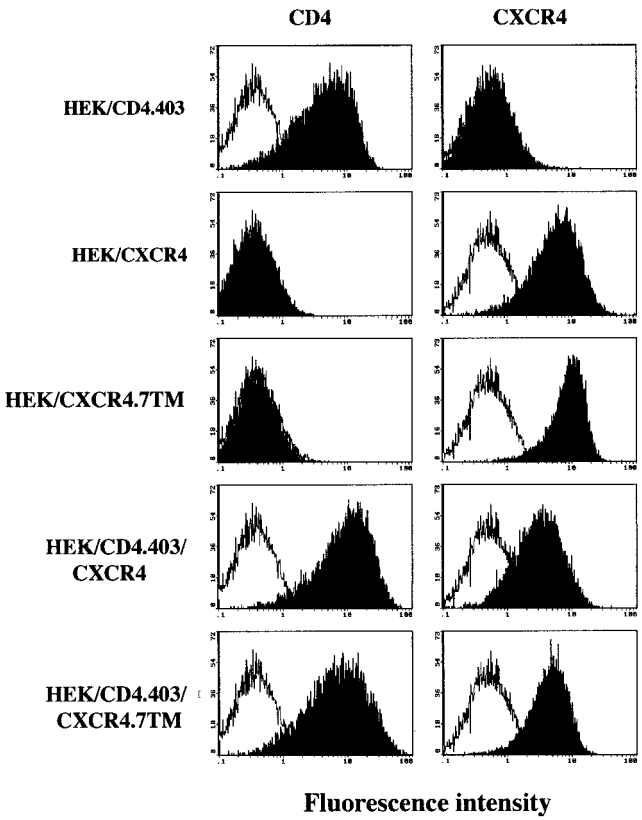


FIG. 1. Expression of CD4.403 and CXCR4 or CXCR4.7TM in transfected HEK cells. The cell surface expression of CD4.403 and various forms of CXCR4 in HEK/CD4.403, HEK/CXCR4, HEK/CXCR4.7TM, HEK/CD4.403/CXCR4, and HEK/CD4.403/CXCR4.7TM was detected by flow cytometry. Cells were incubated with medium alone (white histograms) or medium containing the anti-CD4 (left) or anti-CXCR4 (right) mAbs at 10 μg/ml (black histograms). Bound mAb was detected by a FITC-labeled GAM Ig. The fluorescence intensity was recorded in the log mode on an EPICS XL4 cytofluorometer.

infection, the HIV-1 $p24^{gag}$ protein was measured in HEK, HEK/CD4.403, and HEK/CD4.403/CXCR4 cells. High amounts of virus particles were detected only in doubly transfected cells, indicating that transfected CXCR4 allows efficient HIV-1 entry (Fig. 2A). Second, we analyzed the capability of transfected CXCR4 to transduce a signal in HEK/CD4.403/CXCR4 cells using SDF-1α, the natural ligand of CXCR4. SDF-1α is known to induce tyrosine phosphorylation of the protein tyrosine kinase Pyk2 and to activate the ERK signaling pathway in T cells through CXCR4. As HEK cells exhibit low expression levels of endogenous Pyk2 (Della Rocca *et al.,* 1997; Xiong and Parsons, 1997), a large amount (500 μg) of protein from the cell lysate was used to perform this assay. Under such experimental conditions, Pyk2 phosphorylation was detected after treatment of HEK/CD4.403/CXCR4 cells with PMA-Ionomycin and SDF-1α. In contrast, RANTES, a ligand of CCR5 HIV-1 coreceptor, did not trigger Pyk2 phosphorylation, indicating that the signal observed was specific (Fig. 2B). We also demonstrated in Fig. 2C that SDF-1α activates ERK in HEK/CD4.403/CXCR4 cells.

Altogether, our results corroborate previous observations by others (Lee *et al.,* 1998; Wang *et al.,* 1998), indicating that this model of CXCR4-transfected cells is appropriate for studying signal transduction events induced by binding of CXCR4 natural ligand, SDF-1α. Since we found CXCR4 expressed on HEK cells to serve as coreceptor for HIV-1 entry, this model should be also appropriated to investigate signals triggered by HIV-1 envelope binding to CXCR4.

Role of CXCR4 in HIV-1 induction of syncytia

It is generally accepted that HIV-1-induced syncytia formation involves interactions between HIV-1 $gp120^{env}$ and CD4 (Sodroski *et al.,* 1986). Here, we investigated the role of CXCR4 in this process, using cocultures of transfected or untransfected HEK cells with 8.E5 cells. The HIV-1 provirus (IIIB) expressed in 8.E5 cells is defective for reverse transcriptase; consequently, 8.E5 cells are unable to produce infectious viral particles but express $gp120^{env}$ molecules at their surface (Fig. 3). Syncytia formation was strongly and rapidly induced when HEK/CD4.403/CXCR4 and HEK/CD4.403/CXCR4.7TM cells were cocultured with 8.E5 cells, while only a few,

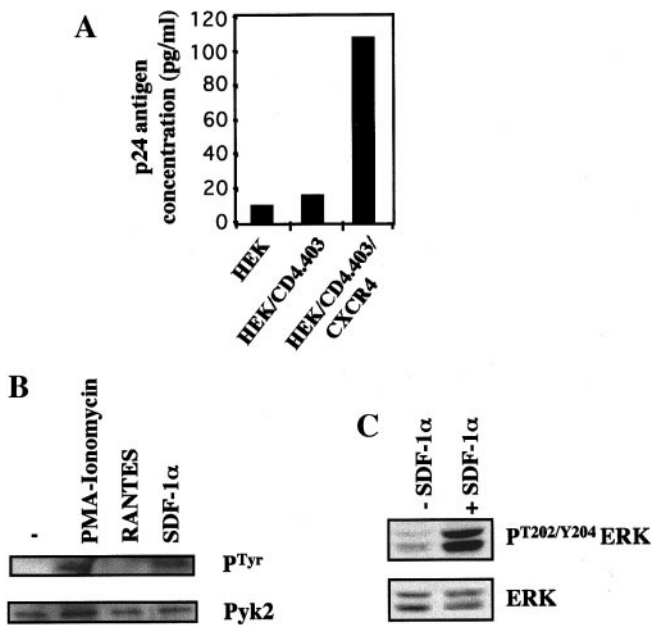


FIG. 2. Ability of transfected CXCR4 in HEK cells to allow viral entry and to transduce signals. (A) Measurement of HIV-1 $p24^{gag}$ protein in culture supernatants of HEK, HEK/CD4.403, and HEK/CD4.403/CXCR4 cells 4 days after exposure to an X4 isolate of HIV-1 (Lai). (B) Analysis of Pyk2 phosphorylation in response to chemokines. HEK/CD4.403/CXCR4 cells were incubated with PMA–Ionomycin for 15 min at 37°C and 500 nM RANTES (CCR5 ligand) or SDF-1α (CXCR4 ligand) at 37°C for 30 s. Samples were immunoprecipitated with anti-Pyk2 antibody and blotted with anti-PTyr (top) or anti-Pyk2 antibody (bottom). (C) HEK/CD4.403/CXCR4 cells were stimulated by SDF-1α for 1 min at 37°C and lysed. Phosphorylation of ERK was detected on Western blots of cell lysates by immunodetection using an anti-phosphoERK antibody (top). (Bottom) A loading control using an anti-ERK antibody.

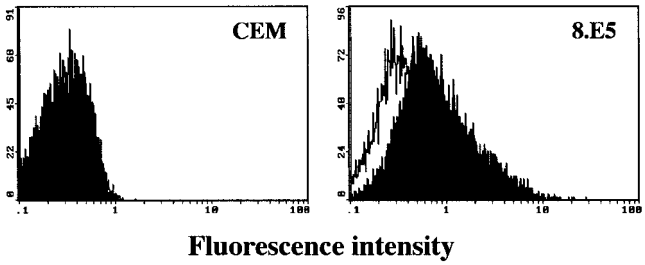


FIG. 3. Cell surface expression of gp120^{env} in human cell lines. Virus-free CEM (left) and HIV-1-positive 8.E5 (right) cells were incubated with medium alone (white histograms) or medium containing the anti-gp120 mAb 110.4 (black histograms). Bound mAb was detected as described in Fig. 1.

small syncytia were found after coculture of HEK/CD4.403 with 8.E5 cells (Fig. 4, left). No syncytia were observed when cells expressing CXCR4 alone were in contact with 8.E5 cells. This result indicates that syncytia formation not only requires HIV-1 gp120^{env}–CD4 interaction but also HIV-1 envelope proteins to bind the extracytoplasmic domains of CXCR4. Suramin, a compound that binds with a high affinity to the V3 loop of HIV-1 gp120^{env} (Fantini *et al.,* 1997), completely inhibited syncytia formation (Fig. 4, right). Similarly, the monoclonal antibodies MAB173 and ST4 at 100 and 50 μg/ml, respectively, inhibit syncytia formation (data not shown).

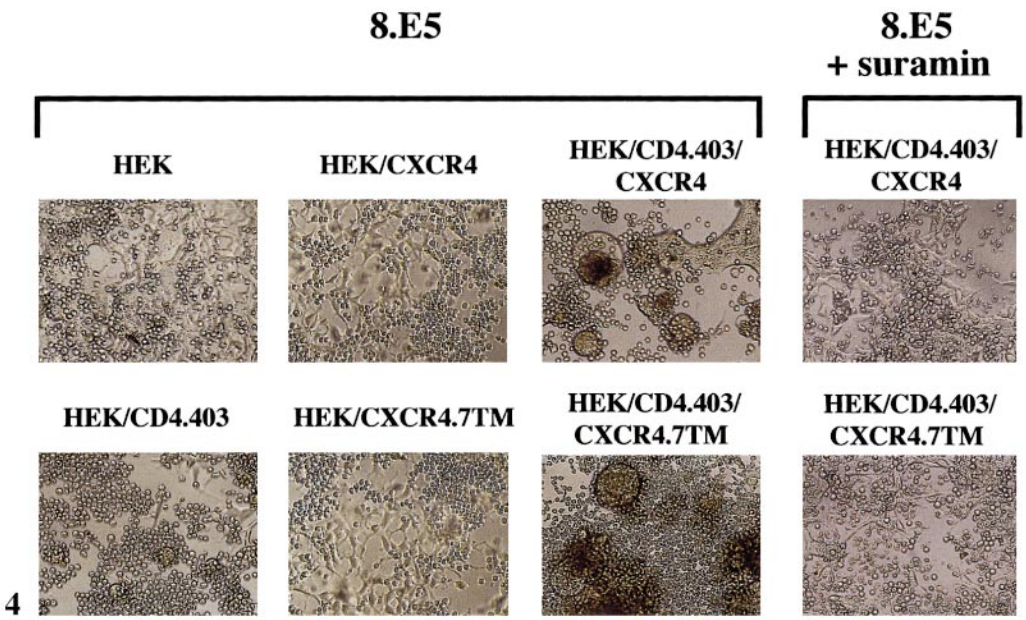
Role of CXCR4 in HIV-1-induced apoptosis

Apoptosis was studied in HEK cells expressing CD4.403 and different forms of CXCR4 after coculture with 8.E5 cells. Since DNA fragmentation is one hallmark of apoptosis, we analyzed the chromatin breakdown process using either staining of nuclei with the fluorescent dye Hoechst or double-stranded DNA break detection by incorporation of biotin-labeled deoxynucleotides on the 3′-OH termini (TUNEL assay). First, time course experiment indicated that apoptosis was induced in doubly transfected HEK cells by coculture with 8.E5 cells for 3 days and the highest percentage of apoptotic cells was observed after 4 days of coculture. No apoptosis was observed when doubly transfected cells were exposed to iHIV. To verify that apoptosis was specifically induced by a viral gene product, similar experiments were performed with the parental uninfected CEM cells, and no apoptosis was found (Fig. 5A). To eliminate a putative role of soluble factors such as Tat, HEK/CD4.403/CXCR4 cells were incubated with the supernatant of 8.E5 cells cultured to a high density; this culture supernatant did not induce apoptosis (data not shown). Next, the different transfected or untransfected HEK cells were cocultured with 8.E5 cells for 4 days. 8.E5 cells triggered apoptosis in HEK/CD4.403/CXCR4 and HEK/CD4.403/CXCR4.7TM but not in singly transfected cell clones or untransfected HEK cells (Fig. 5B), indicating that both CD4 and CXCR4 are required for HIV-1-induced apoptosis. Under these experimental conditions, CEM cells (Fig. 5B) or iHIV (data not shown) did not trigger significant apoptosis in either transfected or untransfected HEK cells. Representative photographs are shown in Fig. 6. This programmed cell death was inhibited in a dose-dependent manner by an anti-CXCR4 mAb (MAB173) and by the mAb ST4, directed against the gp120-binding site on CD4 (Fig. 7A). At the same concentrations, isotype controls did not show any apoptosis inhibition (data not shown). Inhibition of programmed cell death by MAB173 and ST4 in HEK/CD4.403/CXCR4 and HEK/CD4.403/CXCR4.7TM clones were similar. Furthermore, SDF-1α at 500 ng/ml inhibited 8.E5-induced apoptosis of doubly transfected HEK cells (Fig. 7B). It is worth noting that SDF-1α alone did not induce apoptosis in our cellular system (data not shown). In addition, suramin, a G protein inhibitor (Beindl *et al.,* 1996) that binds to the V3 loop of HIV-1 gp120^{env} (Fantini *et al.,* 1997), showed dose-dependent inhibition of apoptosis in HEK/CD4.403/CXCR4 and HEK/CD4.403/CXCR4.7TM cells (Fig. 8A). At 50 μM suramin, the inhibition of 8.E5-induced apoptosis was complete, as measured by Hoechst staining (Fig. 8B) and TUNEL assay (Fig. 8C).

These results indicate that (i) cell surface expression of both exogenous CXCR4 and the extracellular domain of CD4 are needed to observe apoptosis in transfected HEK cells, (ii) the cytoplasmic tail of CXCR4 is not required, (iii) the viral glycoprotein involved is gp120^{env}, since apoptosis is inhibited by ligands of the gp120-binding site on CD4, of CXCR4, and of the gp120^{env} V3-loop, and (iv) gp120^{env} expressed at the cell surface, but not iHIV, triggers programmed cell death in doubly transfected HEK cells.

Search for mediators of CXCR4-driven apoptotic signaling

The envelope of X4 isolates of HIV-1 has previously been found able to activate Pyk2, a tyrosine kinase with multiple potential effector pathways. Among them are the stress pathways that include p38^{MAPK} and SAPK/JNK, as well as numerous pathways that target the transcription factors cAMP responsive element binding protein (CREB) and activating transcription factor 1 (ATF1). CREB phosphorylation on Ser 133 potentiates its ability to activate transcription, while the activation of the kinases SAPK/JNK and p38^{MAPK} occurs via their phosphorylation on a Thr–Xaa–Tyr motif. Therefore their recognition by antibodies specific for the phosphorylated forms reflects their state of activation. Using immunoblotting, we investigated the putative consequences of HIV-1 envelope binding to CXCR4 on p38^{MAPK}, JNK, and CREB activation. To this end, we studied the phosphorylation of these molecules after HEK/CD4.403 and HEK/CD4.403/CXCR4 cells had been cocultured with 8.E5 cells. We found that



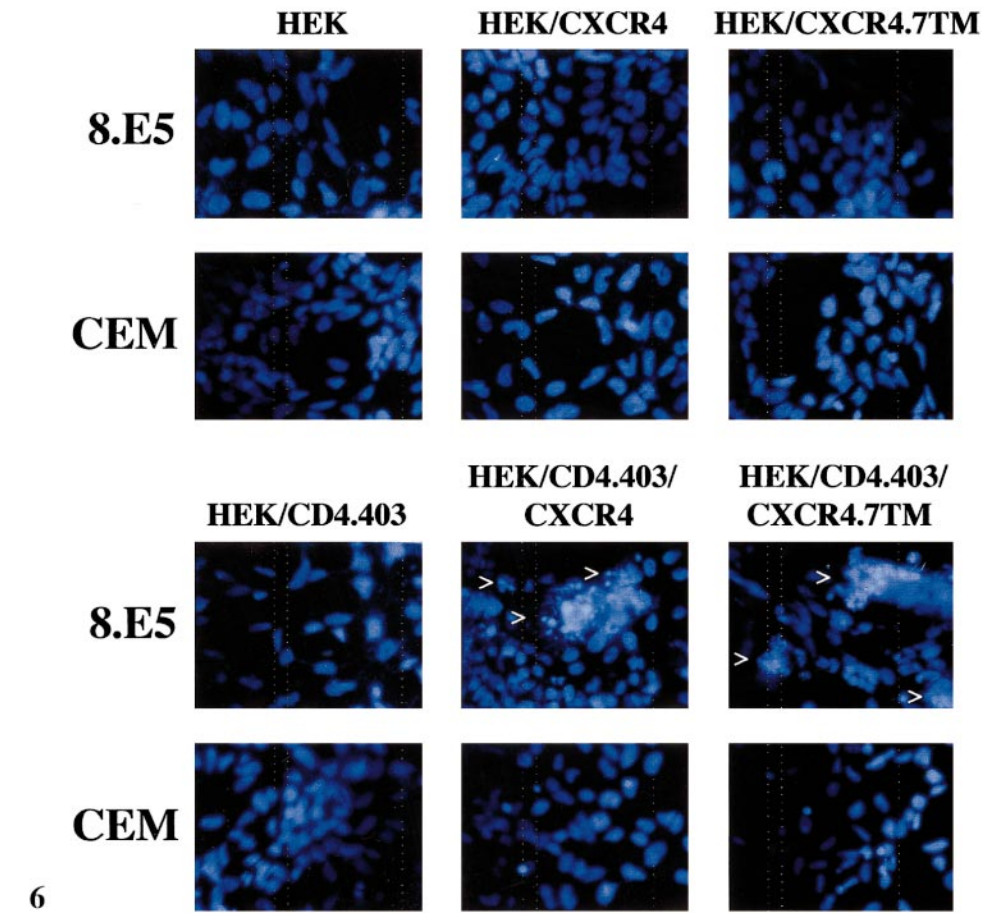


FIG. 4. Involvement of CXCR4 and CD4.403 in syncytia formation. Phase-contrast photomicrographs of syncytia formed when HEK, HEK/CD4.403, HEK/CXCR4, HEK/CXCR4.7TM, HEK/CD4.403/CXCR4, and HEK/CD4.403/CXCR4.7TM cells were cocultured for 4 days with 8.E5 cells in the presence or in the absence of suramin (original magnification, 20×).

FIG. 6. Apoptosis analysis by Hoechst staining. Transfected or untransfected HEK cells were cocultured for 4 days with 8.E5 or CEM cells. HEK cells were fixed, stained with Hoechst solution, visualized by microscopy with UV illumination, and photographed (original magnification, 40×). Apoptotic cells are indicated by arrowheads.

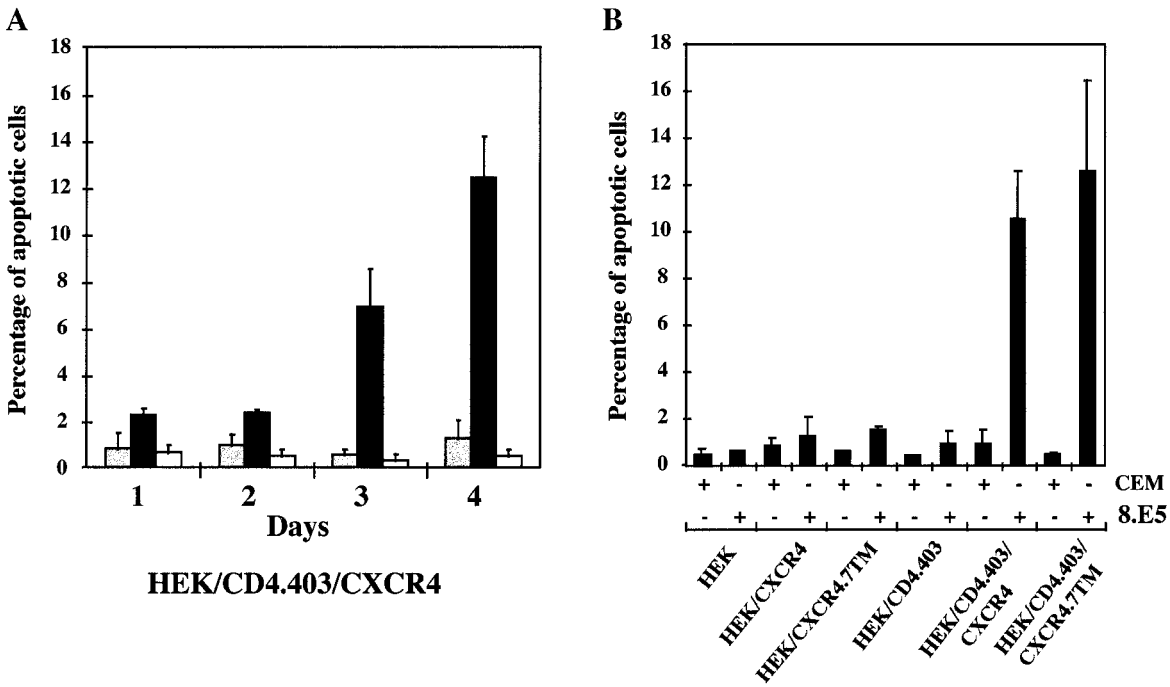


FIG. 5. Percentage of apoptotic cells in HEK transfected or untransfected clones cocultured with 8.E5 or CEM, or incubated with heat inactivated HIV-1 (iHIV). (A) Doubly transfected HEK cells were cocultured with 8.E5 (black histogram) or CEM cells (white histogram), or incubated with iHIV (gray histogram) for 1 to 4 days. (B) Transfected or untransfected HEK cells were cocultured for 4 days with 8.E5 or CEM cells. HEK cells were fixed, stained with Hoechst solution, and examined under an inverted microscope with UV light. Results (means ± SEM% apoptotic cells/field) are from at least four independent experiments.

p38^{MAPK}, SAPK/JNK and CREB were not activated under these experimental conditions (Fig. 9A). In contrast, phosphorylation was induced by PMA/ionomycin and anisomycin. Interestingly, phosphorylation of ATF1 was decreased in HEK transfected with CD4.403 and CXCR4, relative to CD4.403 transfected cells. Since Pyk2 phosphorylation induced after binding of HIV-1 envelope to CD4$^+$ cells was sensitive to pertussis toxin (PTX) treatment (Davis *et al.,* 1997), we also investigated the ability of this toxin to inhibit HIV-1 gp120^{env}-induced apoptosis. PTX at 500 ng/ml, a concentration previously shown to completely inhibit the calcium mobilization induced by SDF-1α in transfected adherent cells expressing CXCR4 (Coudronnière *et al.,* 1998), did not affect HIV-1 gp120^{env}-induced apoptosis (Fig. 9B). Previous studies demonstrated that PI-3K and its metabolic products play an important role in signaling pathways related to chemotaxis (Carpenter and Cantley, 1996) and that PI-3K was activated after SDF-1α binding to CXCR4 (Ganju *et al.,* 1998). Accordingly, the PI-3K inhibitor wortmannin was found to partially inhibit SDF1-α-induced cell migration (Ganju *et al.,* 1998). In our cellular model, wortmannin at 100 nM did not inhibit cell surface-expressed gp120^{env}-induced apoptosis, excluding a role for PI-3K (Fig. 9B). Interestingly, the broad spectrum caspase inhibitor z-VAD.fmk inhibited this programmed cell death, indicating that caspases are implicated in this apoptotic pathway (Fig. 9B). To better understand which caspases are involved in this process, we used more specific caspase inhibitor peptides at various concentrations: z-YVAD.fmk, which inhibits caspases 1 and 4, and z-DEVD.fmk, an inhibitor of caspases 3, 6, 7, 8, and 10. First, these caspase inhibitors were added at a 50 μM concentration in Fas- and etoposide-treated transfected HEK cell culture to control their efficiency at inhibiting apoptosis

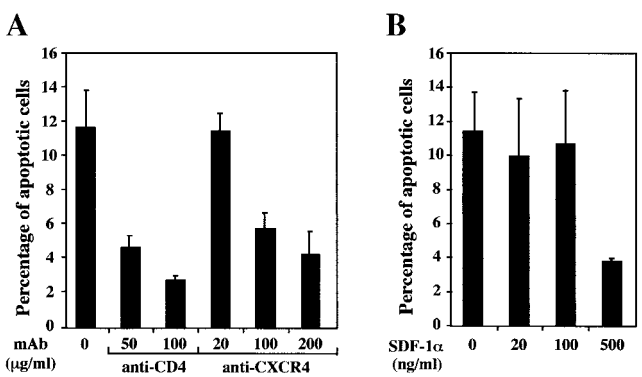


FIG. 7. Apoptosis inhibition of doubly transfected HEK cells cocultured with 8.E5 cells by anti-CD4 and anti-CXCR4 antibodies and SDF-1α. HEK cells expressing CD4.403 and CXCR4 were cocultured for 4 days with 8.E5 cells in the presence or the absence of increasing concentrations of (A) monoclonal antibodies directed against CD4 (ST4) or CXCR4 (MAB173) or (B) recombinant SDF-1α. Percentage of apoptotic cells was determined as described in legend to Fig. 5.

induced by these reagents. z-VAD.fmk, z-DEVD.fmk, and z-YVAD.fmk blocked Fas-induced apoptosis, whereas only z-VAD.fmk was able to inhibit etoposide-mediated apoptosis (Fig. 9C). Each caspase inhibitor was also added at different concentrations in this cellular system. z-VAD.fmk (50 μM), 25 μM z-YVAD.fmk, and z-DEVD.fmk completely blocked apoptosis of transfected HEK cells induced by the anti-Fas antibody (clone CH11). Interestingly, 25 μM z-VAD.fmk did not inhibit etoposide-mediated apoptosis, while this concentration was still able to strongly inhibit (64.6%) CH11-induced HEK cell death (data not shown). Next, the ability of caspase inhibitors to prevent gp120^{env}-induced apoptosis of transfected HEK cells was studied. Doubly transfected HEK cell apoptosis was inhibited by z-VAD.fmk and z-DEVD.fmk at concentrations of 50 and 100 μM, while z-YVAD.fmk inhibits very weakly at 100 μM (Fig. 9D).

Although gp120^{env} binding to CXCR4 induces neither the p38^{MAPK} nor the SAPK/JNK, engagement of HIV-1 gp120^{env} with CXCR4 triggers a death program that involves caspases, probably one of the caspase 3-like proteases.

DISCUSSION

The aim of this study was to investigate HIV-1-induced apoptosis through CXCR4 in cells that express CD4 molecules capable of binding to gp120^{env} but unable to transduce a signal on its own. Chemokine receptors, as CD4, are cell surface molecules that play major roles in T-cell signal transduction and can also deliver signals after engagement with HIV-1, leading to T-cell activation or apoptosis. There is therefore a need for a better understanding of signal transduction pathways activated by HIV-1 to determine which pathways are induced through CD4 and which ones can be ascribed to G protein-coupled receptors. Moreover, it is of major importance to distinguish between pathways which trigger cell activation from those which lead to apoptosis. To dissect the signal specifically transducted by CXCR4 after gp120 binding, without any interference with the pathways activated through CD4, we used a model cell system in which the external part of the CD4 molecule is present to allow subsequent gp120 binding on CXCR4 but in which the cytoplasmic tail is absent to avoid the interaction with CD4 downstream signaling cascades.

The events subsequent to binding of the HIV-1 envelope glycoproteins to CD4 and chemokine coreceptors that lead to HIV-1-mediated syncytia formation are not completely understood. First, syncytia formation was shown to be dependent on the interaction between HIV-1 gp120^{env} on the surface of infected cells and the CD4 receptor molecule of uninfected cells (Dalgleish *et al.,* 1984; McDougal *et al.,* 1986; Sodroski *et al.,* 1986). Subsequent work demonstrated that HIV-1 entry also depends on the presence of specific chemokine receptors. Indeed, syncytia formation was inhibited by anti-CXCR4 monoclonal antibodies (Barbeau *et al.,* 1998) and small molecules which downmodulate CXCR4 (Howard *et al.,* 1998). We directly demonstrate that CXCR4 and the extracytoplasmic domain of CD4 are needed for the formation of multinucleated giant cells after contact with HIV-1 gp120^{env}. Nevertheless, we cannot exclude a role of other cell surface molecules in this HIV-1-mediated syncytia formation as previously postulated by others (Barbeau *et al.,* 1998; Hioe *et al.,* 1998). There is also some controversy in determining the role played by syncytia in HIV-1-induced cell death. This point will be addressed later in this discussion.

We and several others have shown that while the cytoplasmic tail of CD4 is needed for signal transduction induced by the HIV-1 envelope leading to AP-1 and NF-kB activation, it is necessary neither for gp120^{env} binding nor for syncytia formation (Bedinger *et al.,* 1988; Benkirane *et al.,* 1994; Golding *et al.,* 1993; Poulin *et al.,* 1991; Tremblay *et al.,* 1994). Furthermore, cells expressing truncated forms of CD4 that lack the cytoplasmic domain (CD4.401, CD4.403) can still undergo apoptosis (Guillerm *et al.,* 1998; Jacotot *et al.,* 1997), suggesting that another HIV receptor may be involved in this process. The present study demonstrates that CXCR4 is responsible for apoptosis induced by the gp120 envelope glycoprotein of X4 HIV-1 isolates and that this cell death is not dependent on syncytia formation. Although there is some controversy as to the conformation of gp120^{env} needed to induce cell death, membrane expression of HIV envelope glycoproteins seems necessary to trigger apoptosis in CD4 cells (Corbeil and Richman, 1995; Guillerm *et al.,* 1998; Laurent-Crawford *et al.,* 1993). As a model system, we used 8.E5 cells, which express a significant number of HIV-1 gp120^{env} at their surface (without producing infectious virions), to induce apoptosis in CD4.403 and CXCR4-transfected HEK cells. In parallel, we analyzed the putative role of heat-inactivated HIV (iHIV) in inducing apoptosis.

Only the cells doubly transfected with CD4.403 and CXCR4 or CXCR4.7TM (HEK/CD4.403/CXCR4 and HEK/CD4.403/CXCR4.7TM) underwent apoptosis when they are cocultured with 8.E5 cells. No apoptosis was found when these doubly transfected cells were incubated with recombinant gp120^{env} (data not shown) or iHIV, indicating that gp120^{env} needs to be expressed at the surface of cells to trigger apoptosis. The fact that virus–cell fusion or recombinant gp120^{env} is not able to induce apoptosis might suggest either that gp120^{env} conformation differs between infected cells and iHIV or that signal elicited by adhesion molecules involved in close cell-to-cell contacts could also play a role in apoptosis. Notably, the adhesion molecule ICAM-1 (Rizzuto and Sodroski, 1997) and glycosphingolipids (Hammache *et al.,* 1999) were shown to contribute to cell fusion. Apoptotic doubly transfected CD4.403 and CXCR4 or CXCR4.7TM HEK

cells were found both as single and multinucleated giant cells, indicating that syncytia formation is not essential for induction of apoptosis. Furthermore, cytochalasin B, a compound that induces depolymerization of actin filaments, does not inhibit apoptosis but neutralizes syncytia formation (V.R-H. and M.B-P., unpublished observation). This result is in accordance with a recent paper from J. Blanco and coworkers (Blanco *et al.,* 1999). Nevertheless, syncytia can be involved in virus propagation and thus in disease progression. Since HEK/CD4.403 cells alone did not lead to apoptosis, this indicates that CXCR4 is needed for activating this cell death program. The resistance of HEK/CXCR4 and HEK/CXCR4.7TM cells to apoptotic signals induced by 8.E5 cells also indicates the involvement of CD4 in the initiation of apoptosis. The fact that mAb ST4, directed against the gp120-binding site on CD4, is able to inhibit the apoptotic signal transduction confirms the involvement of CD4 in the death process. Since our model system was selected to avoid CD4 dependent signal transduction, the role played by CD4 probably consists only of increasing the affinity of $gp120^{env}$ for CXCR4 and thus the number of $gp120^{env}$ molecules able to bind CXCR4 (Lapham *et al.,* 1996). This result agrees with those found in other cellular systems, where CXCR4 was shown to transduce apoptotic signals (Herbein *et al.,* 1998; Hesselgesser *et al.,* 1998). Interestingly, each cellular system studied, except neuronal cells, suggests that other surface molecules contribute somehow to HIV-1 envelope-induced apoptosis through CXCR4. Recently, Berndt and collaborators (1998) described that both CD4 and CXCR4 play a role in HIV-1 envelope-induced apoptosis of $CD4^+$ T cells and T cell lines (Herbein *et al.,* 1998; Hesselgesser *et al.,* 1998). We demonstrate here that, in our heterologous cell system, the extracellular domain of CD4 is important but CD4 function is likely limited to allow conformational changes in HIV-1 $gp120^{env}$.

Notably, the cytoplasmic domain of CXCR4 is dispensable for apoptosis. The 18 serine/threonine residues of this cytoplasmic domain, which represent potential targets for phosphorylation by G protein-coupled receptor kinases, second messenger-activated protein kinases, and PKC (Freedman and Lefkowitz, 1996) are thus not involved in this apoptotic pathway. This region of CXCR4 was also implicated in CXCR4 turnover. Indeed, deletion of the C-terminal part of CXCR4 did not affect HIV entry but prevented SDF-1-induced receptor downmodulation (Amara *et al.,* 1997). Nevertheless, Wang and coworkers demonstrated recently that HIV-1 $gp120^{env}$ inhibits the monocyte response to chemokines through CD4-dependent CXCR4 downregulation and showed that the entire CD4 molecule is needed for the internalization of CXCR4 (Wang *et al.,* 1998). It was thus interesting to study the expression of CXCR4 at the surface of HEK/CD4.403/CXCR4 and HEK/CD4.403/CXCR4.7TM cells that have been cocultured for 4 days with 8.E5 cells. Under these experimental conditions, we found no decrease in cell surface expression of CXCR4 wild type or CXCR4.7TM (V.R-H. and M.B-P., unpublished observation). This indicates that CXCR4 is unable to internalize upon HIV-1 $gp120^{env}$ binding when CD4 is expressed with a truncated cytoplasmic tail. The lack of heterologous desensitization makes this a useful cellular model for studying CXCR4 signaling.

To support our findings that $gp120^{env}$ expressed at the surface of 8.E5 cells can specifically induce apoptosis via CXCR4, we demonstrated that the neutralizing mAb MAB173 specific for CXCR4 is able to inhibit the apoptotic pathway in a dose-dependent manner. The same amount of antibody is necessary to neutralize this cell death and to inhibit the syncytia formation. This latter experiment allowed an estimation of the quantity of antibody required to saturate transfected CXCR4 on HEK cells under our experimental cell culture conditions. In the same way, SDF-1α at 500 ng/ml strongly reduced $gp120^{env}$-induced apoptosis. We also tested the effect of suramin as an inhibitor in our assay. This compound binds with a high affinity to the $gp120^{env}$ V3 loop of the viral envelope and blocks HIV-1-induced fusion and entry into CD4-exponent cells. Suramin completely blocked syncytia formation and apoptosis induced by 8.E5 cells in HEK/CD4.403/CXCR4 and HEK/CD4.403/CXCR4.7TM cells independently of the expression level of HIV-1 coreceptors, most likely by inhibiting the binding of $gp120^{env}$ on CXCR4. This would agree with a very recent study demonstrating that apoptosis of primary brain cultures is induced by HIV-1 envelope binding to CXCR4 (Ohagen *et al.,* 1999).

SDF-1 binding to CXCR4 activates the protein kinase ERK, an important mediator of signaling from the membrane to the nucleus for many proliferative agents. Nevertheless, our previous observations (Briant *et al.,* 1998) and the results from others (Popik *et al.,* 1998) did not suggest an involvement of CXCR4 in signals that activate ERK following HIV binding to $CD4^+$ cells. Furthermore, in our cellular model, the ERK pathway inhibitor PD98059 did not block the apoptotic signaling (data not shown). It seemed more likely that the $p38^{MAPK}$ and SAPK/JNK cascades might be involved in HIV-induced apoptosis, since these pathways have been linked to induction of cell death in other systems. These kinases were present in the transfected HEK cells and activated by phosphorylation induced by the stress anisomycin, as well as by PMA coupled with ionomycin. However, neither $p38^{MAPK}$ nor SAPK/JNK were activated under conditions that led to apoptosis, which rules out their role as mediators downstream of the $gp120^{env}$/CXCR4 complex. In confirmation of this, the $p38^{MAPK}$-specific inhibitor SB203580 (at 10 μM) did not inhibit apoptosis (M.B.-P., unpublished data). The same holds true for PI-3K, since the specific inhibitor wortmannin did not affect apoptosis even at high con-

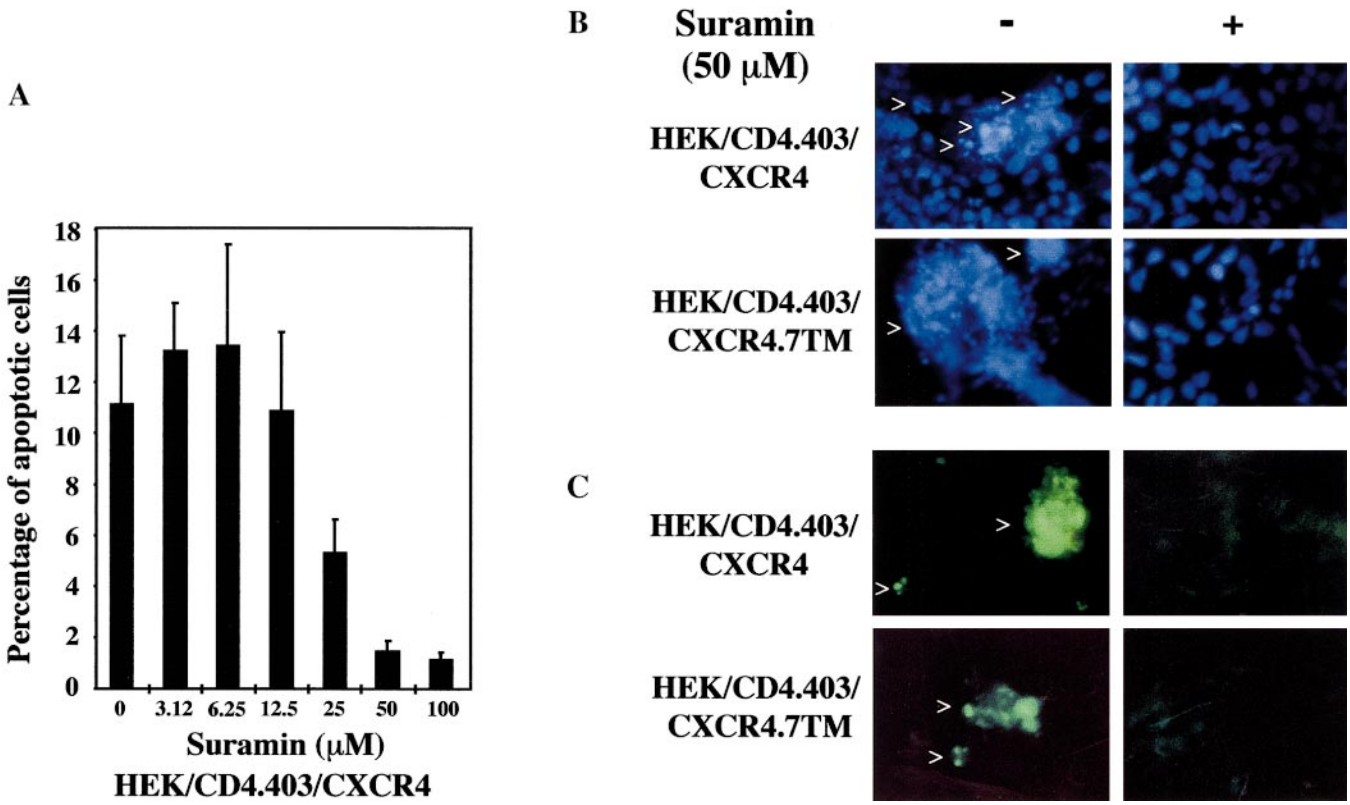


FIG. 8. Apoptosis inhibition of doubly transfected HEK cells cocultured with 8.E5 cells by suramin. Apoptosis of HEK cells expressing CD4.403 and CXCR4 cocultured for 4 days with 8.E5 cells in the presence or the absence of suramin was determined. (A) Different concentrations of suramin were tested and the percentage of apoptotic cells was determined as described under Materials and Methods. (B) Representative photographs of apoptotic cells after Hoechst staining and (C) TUNEL assay. Apoptotic cells are indicated by arrowheads.

centrations. In agreement with these results, Popik and Pitha (Popik and Pitha, 1998) recently reported that rapid phosphorylation of the ERK activating kinase (MEK), ERK, SAPK/JNK, and $p38^{MAPK}$ was not induced by binding of a CXCR4-tropic simian immunodeficiency virus to $CD4^+/CXCR4^+$ cells, although $gp120^{env}$ of an R5 isolate of HIV-1 can stimulate these pathways through CCR5. This demonstrates that these two coreceptors do not transduce equivalent signals. In contrast, SB203580 apparently attenuated $gp120^{env}$-induced neuronal apoptosis, indicating that some neurotoxic processes initiated by $gp120^{env}$ possibly use $p38^{MAPK}$ (Kaul and Lipton, 1999). The transcription factors CREB and ATF1 are useful indicators for multiple intracellular signaling systems. It is well documented for CREB that activating phosphorylation can be mediated by various intracellular second messenger/kinase pathways, such as cAMP/protein kinase A, calcium/calmodulin-dependent kinases II and IV, growth factors/$p90^{Rsk2}$, and stress/(mitogen-activated protein kinase)-activated kinases 2 and 3 (Ghosh and Greenberg, 1995; Ginty, 1997). None of these appears to mediate the $gp120^{env}$/CXCR4 apoptotic signal, since CREB phosphorylation was not induced. In contrast, this signal led to a decrease in phosphorylation of ATF1, therefore indicating that $gp120^{env}$ has induced some, still unidentified, intracellular signaling pathway possibly involved in apoptosis. Its role in the programmed cell death will be established only upon its characterization.

As the caspase family of cysteine proteases are important mediators of apoptosis, we analyzed the ability of caspase inhibitor peptides to block $gp120^{env}$-induced apoptosis. The programmed cell death was inhibited by z-VAD.fmk, indicating that an important stage of this apoptotic process involves caspases. Furthermore, z-DEVD.fmk protected cells from apoptosis, indicating that this process requires activation of caspase-3 (-like) proteases. On the other hand, caspase-1 was not involved in $gp120^{env}$-mediated apoptosis because z-YVAD.fmk failed to markedly inhibit this cell death program, whereas it blocks anti-Fas-induced apoptosis. Interestingly, caspase 3 activation also seems to be correlated with changes in G protein-coupled signaling through CXCR4 during HIV-1-associated dementia (Zheng *et al.,* 1999). Further investigation is needed to identify the transduction pathways that link CXCR4 to caspase activation and subsequent cell death.

Our CD4/CXCR4 transfected HEK cell model has allowed a fine dissection of signals transducted through

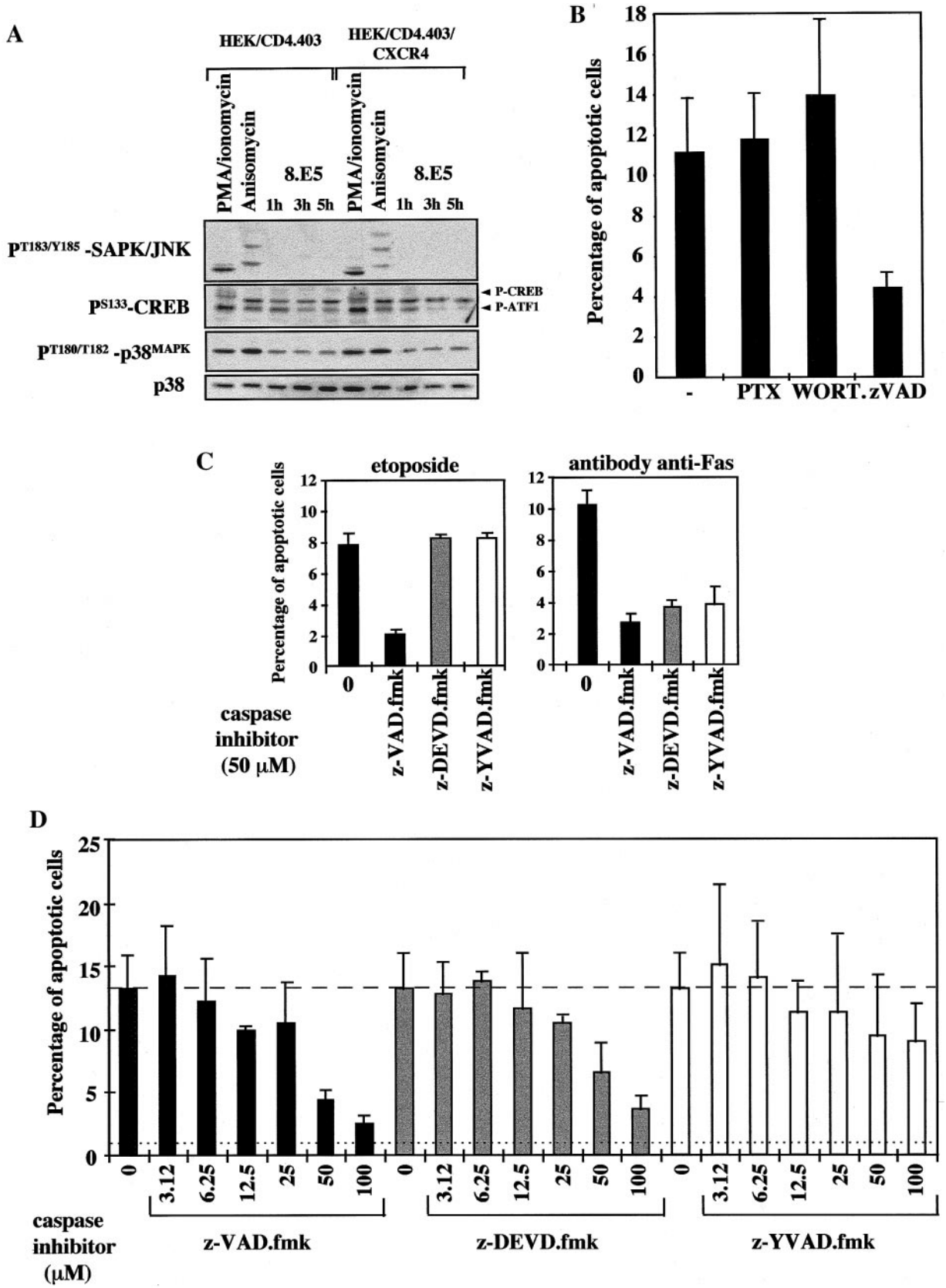


FIG. 9. CXCR4 engagement by gp120^{env} triggers caspase activation. (A) HEK/CD4.403 and HEK/CD4.403/CXCR4 cells were stimulated with 8.E5 cells for the times (1 h, 3 h, or 5 h) indicated above the lanes. To control for inducibility, the cells were also treated with 50 ng/ml anisomycin or PMA/ionomycin as indicated. Cell lysates (15 μg per lane) were electrophoresed on SDS–PAGE minigels, transferred to PVDF, and immunodetected with antibodies specific for the phosphorylated versions of the proteins shown at the left of each panel. A loading control was added using an anti-p38

CXCR4 itself following engagement with HIV-1 gp120^{env} and provides direct evidence that CXCR4 is involved in HIV-1 gp120^{env}-induced apoptosis. These data, however, have to be further confirmed in more physiological systems because signaling pathways in HEK cells are likely not identical to T cells. Nevertheless, very recently, we demonstrated that CXCR4 present on CD4$^+$ human T cell lines plays a major role in HIV-1-induced apoptosis of these cells when cocultured for 2-days with gp120^{env}-expressing adherent cells, indicating that CXCR4 is clearly involved in HIV-1 gp120^{env}-induced apoptosis of CD4$^+$ T cells (Biard-Piechaczyk *et al.,* 1999). This observation probably has important physiological consequences. HIV preference for CCR5 versus CXCR4 undergoes a characteristic change during the course of infection and disease progression; the decline in immune system function coincides with the appearance of more aggressive viruses that use CXCR4 as receptor (Connor *et al.,* 1997; Unutmaz and Littman, 1997). One hypothesis for the increased pathogenicity of X4 strains could be that, after infection with R5 strains, there is first a CD4/CCR5-dependent cell activation to enhance the propagation of the virus, and the switch to CXCR4 coreceptor would correspond with the ability to trigger apoptotic signals and a rapid decline of CD4$^+$ T cells. Therefore, the activation or alteration of the signaling pathways induced by binding of HIV-1 to their receptor and/or coreceptor obviously will have important consequences for HIV-induced pathogenicity, a notion that bears further study.

MATERIALS AND METHODS

Antibodies and reagents

Anti-CD4 monoclonal antibodies (mAb) BL4 and ST4 were kindly provided by M. Hirn (Coulter-Immunotech, Marseille, France) and D. Carriere (Sanofi-Recherche, Montpellier, France), respectively. Anti-CXCR4 mAb (MAB173), RANTES and SDF-1α were purchased from R&D Systems (R&D Systems Europe, Ltd., Abingdon, UK). Anti-gp120 110.4 mAb was obtained from Genetic Systems. Anti-human Fas antibody (clone CH11) was purchased from Upstate Biotechnology (Euromedex, Souffelweyershem, France). Anti-Pyk2 mAb was purchased from TEBU (Le Perray en Yvelines, France). Culture supernatants of 4G10 and PY72 hybridoma were a kind gift from N. Taylor (IGMM, Montpellier). Peroxidase-coupled goat anti-mouse Ig NA 931 was purchased from Amersham (Les Ullis, France). FITC-labeled Fab′2 goat anti-mouse (GAM) immunoglobulin (Ig) was purchased from Immunotech. Rabbit antibodies specific for phosphorylated, activated versions of ERK, SAPK/JNK, p38, and CREB and for unphosphorylated proteins ERK and p38 were obtained from New England Biolabs (Ozyme, Saint Quentin en Yvelines, France). Peroxidase-coupled goat anti-rabbit antiserum was purchased from Sigma-Aldrich (L'Isle D'Abeau Chesnes, France). Protein G–Sepharose was purchased from Pharmacia (Pharmacia Biotech, Saclay, France). Suramin was purchased from TEBU. *Bordetella pertussis* toxin (PTX) was purchased from Calbiochem (France Biochem, Meudon, France). Wortmannin and etoposide were purchased from Sigma-Aldrich, z-Val–Ala–Asp (OMe)–CH_2F (z-VAD.fmk), z-Tyr–Val–Ala–Asp (OMe)–CH_2F (z-YVAD.fmk), and z-Asp(OCH_3)–Glu(OCH_3)–Val–Asp(OMe)–CH_2F (z-DEVD.fmk) were purchased from Calbiochem. Geneticin (G418) and Zeocin were purchased from Gibco-Life Technologies (Eragny, France) and Invitrogen (Abingdon, UK) respectively.

Oligonucleotides

Cloning oligonucleotides. CD4 oligonucleotide primers were 5′CD4 (5′TCG CCG GAA TTC TAC TGC TCA GCC CCT TCC TC-3′) and 3′403 (5′-CCG ATG CTC GAG TTA TGC TTG GCG CCT TCG GTG CC-3′). CXCR4 oligonucleotide primers were 5′Fus BABE (5′-GTG CAC GAA TTC GGC TTA AGT GAC GCC GAG-3′), 3′Fus (5′-CCG ATG GTC GAC TTA CTT GTC ATC GTC GTC CTT GTA GTC GCT GGA GTG AAA ACT TGA AGA-3′), and 3′7TM (5′-CCG ATG GTC GAC TTA CTT GTC ATC GTC GTC CTT GTA GTC GGC TCC AAG GAA AGC ATA GAG-3′). The two 3′ primers used to amplify the CXCR4 gene contain the sequence of the Flag epitope.

Sequencing oligonucleotides. T7 oligonucleotide was provided with the sequencing kit. CD4.1 (5′-TAC ATC TGT GAA GTG GAG-3′), CD4.2 (5′-GAA AAG CTG ACG GGC AGT-3′), CD4.3 (5′-CCT AAG CTG ATG CTG AGC-3′), Fus1 (5′-ACT ATT CCC GAC TTC ATC-3′), Fus2 (5′-CAC

antibody. Two major species of SAPK/JNK are detected, p46 and p54, while all p38^{MAPK} isoforms comigrate under these gel conditions. The phospho-Ser133 CREB antiserum also recognizes the transcription factor ATF1 because the peptide used to generate this serum spans a region highly conserved between these two proteins. (B) Percentage of apoptotic cells analyzed after HEK/CD4.403/CXCR4 cells had been cocultured for 4 days with 8.E5 cells in medium alone (−) or in the presence of pertussis toxin (PTX) at 500 ng/ml, wortmannin (WORT.) at 100 nM, and z-VAD.fmk at 50 μM. (C) Percentage of apoptotic cells studied after incubation of doubly transfected HEK cells with 2.5 μg/ml anti-Fas antibody (clone CH11) or 10 μM etoposide for 7 h in the presence or the absence of 50 μM z-VAD.fmk, z-DEVD.fmk, and z-YVAD.fmk to evaluate the activity of caspase inhibitors. (D) Percentage of apoptotic cells studied after HEK/CD4.403/CXCR4 cells had been cocultured for 4 days with 8.E5 cells in the presence or the absence of increasing concentrations of z-VAD.fmk (black histograms), z-DEVD.fmk (gray histograms), or z-YVAD.fmk (white histograms). For easy reading of the figure, a broken line illustrates the level of doubly transfected HEK cell apoptosis observed in absence of inhibitor. Dotted line corresponds to the background of doubly transfected HEK cell apoptosis observed when 8.E5 cells were replaced by CEM.

AGT CAT CCT CAT CCT-3′), Fus seq (5′-CTG GTA CTT TGG GAA CTT C-3′).

All these oligonucleotides were purchased from Eurogentec (Seraing, Belgium).

CD4 and CXCR4 expression vectors

The CD4.403 DNA fragment was amplified by PCR using the 5′CD4/3′403 oligonucleotide primer pair and the retroviral vector pMV7 containing the full-length CD4 cDNA obtained from Q. Sattentau (CIML, Marseille). The CD4.403 PCR DNA fragment was cloned in the pcDNA3 expression vector (Invitrogen). The CXCR4 fragment was amplified by PCR using the 5′Fus BABE/3′Fus oligonucleotide primer pair and, as template, the pBABE. Fusin vector was obtained through the NIH AIDS Research and Reference Reagent Program from N. Landau (Aaron Diamond AIDS Research Center, The Rockefeller University). This fragment was cloned in the pcDNA3 Zeo expression vector (Invitrogen). The CXCR4.7TM PCR DNA fragment was amplified by PCR using the 5′Fus BABE/3′7TM oligonucleotide primer pair, and was cloned in the pcDNA3 Zeo. Before use, all inserts were sequenced on an Applied Biosystems Model 373A automated sequencer, using *Taq* polymerase and dye terminator.

Cells

The CEM T cell line was provided from the American Type Culture Collection (Bethesda, MD). The 8.E5 cell line, a CEM-derived T cell line containing a single integrated copy of X4 HIV-1 was provided by F. Barré-Sinoussi (Institut Pasteur, Paris). Cells were cultured in RPMI 1640 medium supplemented with 1% penicillin–streptomycin (PS) antibiotic mixture, 1% Glutamax, and 10% fetal calf serum (FCS; Gibco-Life Technologies) to a density of 5×10^5 cells/ml in a 5% CO_2 atmosphere. Human embryonic kidney (HEK-293) cell line transformed by sheared human adenovirus type 5 DNA was provided by P. Boulanger (Institut de Biologie, Montpellier) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% PS antibiotics, 1% Glutamax, and 10% FCS. HEK cells (10^7) were transfected with 20 μg of pcDNA3 CD4.403, pcDNA3 Zeo CXCR4, or pcDNA3 Zeo CXCR4.7TM plasmid using the classical calcium phosphate precipitation procedure (Sambrook *et al.,* 1989). Clones able to grow in the presence of the selection agent (G418 or Zeocin) were tested by flow cytometry for surface expression of CD4 or the different forms of CXCR4. HEK cells stably transfected with the pcDNA Neo CD4.403 vector were supertransfected by a second vector using a similar strategy, in order to select cells coexpressing CD4.403 and different forms of CXCR4. Several clones of each type were selected.

Detection for HIV-1 infection

Cells (5×10^5) were incubated for 2.5 h at 37°C with 100 μl of HIV-1 (Lai strain; X4 isolate) at a concentration of 1000 × 50% tissue culture infective dose ($TCID_{50}$) per ml. Thereafter, cells were washed five times and cultured in 24-microwell plates. The amount of HIV-1 produced was monitored 4 days after infection by measuring $p24^{gag}$ protein with a commercial antigen capture ELISA assay (Coulter, Coultronics, Margency, France).

ERK phosphorylation induced after SDF-1α-binding on CXCR4

Twenty-four hours before cell activation, HEK/CD4.403/CXCR4 cells were cultured in medium with 0.5% FCS to lower the basal level of protein activation. Cells were stimulated with SDF-1α at 500 nM for 1 min at 37°C, washed twice in PBS containing sodium fluoride (NaF) 10 mM, sodium orthovanadate (Na_3VO_4) 1 mM, β-glycerophosphate (βGP) 20 mM, and *p*-nitrophenylphosphate (PNPP) 10 mM, and lysed in Tris–HCl 50 mM (pH 8), Triton X-100 1%, NaCl 100 mM, $MgCl_2$ 1 mM, benzamidine 2 mM, leupeptine 2 μg/ml, PMSF 150 μM containing NaF, Na_3VO_4, βGP, and PNPP as above (Hipskind *et al.,* 1994). Cell lysates were electrophoresed in 10% SDS–PAGE and blotted to PVDF membranes (Millipore). Membranes were then blocked in Tris-buffered saline (TBS), 5% BSA, 0.05% Tween 20 for 1 h at 20°C. Blots were incubated overnight at 4°C with the primary antibody diluted 1/1000 in the blocking buffer. After 30 min of washing in six changes of TBS–Tween, the blots were incubated for 1 h at 20°C with peroxidase-coupled goat anti-rabbit antiserum diluted 1/2000 in TBS–5% milk–Tween. After further washing, the immune complexes were revealed by enhanced chemiluminescence (ECL, NEN) and autoradiographied.

Pyk2 phosphorylation induced by chemokines

HEK/CD4.403/CXCR4 cells were stimulated with phorbol myristate acetate (PMA) at 40 ng/ml and Ionomycin at 1 μM for 15 min, RANTES or SDF-1α at 500 nM for 30 s, washed, and lysed. Each lysate containing 500 μg of protein was precleared by incubation for 1 h with 50 μl of protein G–Sepharose (Sigma). Pyk2 was immunoprecipitated from supernatants for 12 h incubation at 4°C with anti-Pyk2 mAb fixed on protein G–Sepharose. The beads were washed three times in lysis buffer, resuspended in an equal volume of SDS–PAGE sample buffer, and analyzed on SDS–PAGE (8% polyacrylamide) gels. After immunoblotting, the membrane was incubated with the two anti-phosphotyrosine (anti-PTyr) antibodies (4G10 and PY72 mAbs) for 2 h at 20°C. After three washings, the NA 931 mAb was incubated for 30 min at 20°C and the blot was revealed as previously described. After dehybridization, the blot was incubated with the anti-Pyk2 mAb at 2

μg/ml for 2 h at 20°C, washed, and the peroxidase anti-mouse Ig diluted 1/3000 was then added for 30 min at 20°C. The blot was revealed as before.

Activation of p38, SAPK/JNK, and CREB after gp120-binding on CXCR4

After being cocultured with 8.E5 cells, transfectant HEK cells were washed and lysed as previously described. Cell lysates were electrophoresed in 10% SDS–PAGE and blotted to PVDF membranes. Blots were incubated overnight at 4°C with primary antibodies diluted 1/1000 in the blocking buffer. After washings, the blots were incubated for 1 h at 20°C with peroxidase-coupled goat anti-rabbit antiserum diluted 1/2000 and the immune complexes were revealed by enhanced chemiluminescence and autoradiographied.

Flow cytometry

Cells (1×10^5) were incubated for 1 h at 4°C with 50 μl of PBS containing 0.2% BSA (PBS–BSA) or PBS–BSA supplemented with the appropriate mAb at concentrations necessary for saturation of cell surface molecules. After three washes with PBS–BSA, bound mAb was revealed by addition of 50 μl of a 1/50 dilution of fluorescein-conjugated (FITC) GAM Ig (Immunotech). After 30 min staining, cells were washed with PBS–BSA and fluorescence intensity at 543 nm was measured on an EPICS XL4-C cytofluorometer (Coulter, Coultronics, Margency, France).

Determination of syncytia formation

Transfected or untransfected HEK cells were cultured in the presence of 10^5 8.E5 cells for 4 days at 37°C in a 5% CO_2 atmosphere in the absence or in the presence of agents that may interfere with cell lysis. Syncytia formation was monitored by light microscopy.

Detection of apoptosis

Detection of HIV-1-induced apoptosis was monitored by nuclear condensation and DNA fragmentation. Apoptotic adherent cells cultured on slides in 24-well plates were visualized using either Hoechst staining (dye Hoechst 33258, Sigma) or an ApoDETEK kit (Enzo Diagnostics, Farmingdale, NY) for the terminal dUTP nick end-labeling (TUNEL) assay. Briefly, adherent cells were cocultured for 1 to 4 days with 10^5 8.E5 or CEM cells per ml. Then, adherent cells were extensively washed with complete medium to eliminate the T cells in suspension and fixed in 3.7% paraformaldehyde in PBS (pH 7.4) containing 0.1% Triton X-100 for 15 min at 20°C. After two washings with PBS, cells were incubated with either Hoechst solution (Sigma) at 0.2 μg/ml for 30 min at 20°C or the terminal deoxynucleotide transferase and biotin-16–dUTP for 1 h at 37°C followed by incubation with PBS containing avidine-FITC for 30 min at 4°C. After two washings, slides were mounted using Mowiol solution and examined by epifluorescence. Monoclonal antibodies used as inhibitors were preincubated for 1 h with HEK transfected cells before adding 8.E5 cells. The percentage of living and apoptotic cells was recorded in more than 14 random fields containing less than 100 cells per field. The total number of counted cells per slide was always higher than 200 cells. Results are from at least four independent experiments. Apoptosis was also analyzed after incubation of transfected or untransfected HEK cells with heat-inactivated HIV-1 (iHIV) from Lai strain (an X4 isolate of HIV-1). iHIV solution corresponds to $1000 \times 50\%$ tissue culture infective doses ($TCID_{50}$) of infectious virus per ml.

ACKNOWLEDGMENTS

We thank N. Coudronnière for technical assistance and helpful discussions and S. Harrington for editorial assistance. This work was partially supported by Institutional funds from the Centre National de la Recherche Scientifique (CNRS) and grants from the Agence Nationale de Recherches sur le SIDA (ANRS), FRM-SIDACTION program to C.D. and from the Fondation pour la Recherche Médicale, Association pour la Recherche sur le Cancer, and Novartis Inc. to R.A.H.

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