HIV-1 gp120-mediated Apoptosis of T Cells Is Regulated by the Membrane Tyrosine Phosphatase CD45*

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The molecular mechanism of the human immunodeficiency virus type 1 (HIV-1) gp120-induced apoptosis of bystander T cells is not well defined. Here, we demonstrate that CD45, a key component of the T cell receptor pathway, plays a crucial role in apoptosis induced by HIV-1 gp120. We observed that HIV-1 gp120-induced apoptosis was significantly reduced in a CD45-deficient cell line and that reconstitution of CD45 in these cells restored gp120-induced apoptosis. However, expression of a chimeric protein containing only the intracellular phosphatase domain was not able to restore the apoptotic function in the CD45-negative clone, indicating an important role for the extracellular domain of CD45 in this function. The role of CD45 in gp120-induced apoptosis was further confirmed in T cell lines and peripheral blood mononuclear cells using a selective CD45 inhibitor as well as CD45-specific small interfering RNA. We also observed that gp120 treatment induced CD45 association with the HIV coreceptor CXCR4. Further elucidation of downstream signaling events revealed that CD45 modulates HIV-1 gp120-induced apoptosis by regulating Fas ligand induction and activation of the phosphoinositide 3-kinase/Akt pathway. These results suggest a novel CD45-mediated mechanism for the HIV envelope-induced apoptosis of T cells.

The depletion of CD4+ T lymphocytes is a central pathogenic feature of human immunodeficiency virus type 1 (HIV-1)2 infection and is largely responsible for the profound immunodeficiency characteristic of the late stages of HIV disease (1). Multiple mechanisms have been shown to contribute to the HIV-1-associated loss of CD4+ T cells in HIV-1-infected individuals, among which the HIV-1-induced apoptosis of bystander uninfected cells is considered to be the most important (2–5). Several virally encoded proteins, including the envelope protein gp120, Tat, and Vpr, have been implicated in this process (3, 6, 7). Cumulative data demonstrate a major role for the HIV envelope glycoprotein in the death of uninfected lymphocytes (8, 9). The presence of measurable amounts of circulating soluble gp120 in HIV-infected subjects and the accumulation of a high concentration of envelope glycoproteins in lymphoid tissues underscores the potential for the envelope to contribute to T cell dysfunction (10, 11). A number of studies have indicated that HIV-1 gp120-induced apoptosis could represent an exacerbation of activation-induced cell death (12, 13). In fact, circulating T lymphocytes in HIV-1-infected patients are characterized by a high degree of immune activation (14, 15). Although immune activation may modulate HIV-mediated apoptosis, the molecular mechanisms involving the cross-talk between the T cell receptor (TCR) pathway and HIV-1 gp120 and leading to T cell loss are still unclear. Because the protein-tyrosine phosphatase CD45 plays an essential role in TCR signaling (16–18), it seemed reasonable to speculate that it may play a key role in HIV envelope-mediated apoptosis.

CD45 is a transmembrane protein-tyrosine phosphatase expressed on the surface of all nucleated hematopoietic cells and is one of the most abundant glycoproteins on the surface of lymphoid cells (19, 20). It is now widely accepted that, in lymphocytes, CD45 acts as a positive regulator of signal transduction through both T and B cell receptors (21, 22). Studies of CD45-deficient human, CD45-deficient mouse, and CD45 mutant cell lines have demonstrated the essential role of CD45 in lymphocyte development and differentiation (22–24). Recently, it has been shown that CD45 also modulates many other T cell functions, including cytokine- and integrin-mediated signaling pathways as well as signals emanating from the chemokine receptor CXCR4 (25–28). In addition, several distinct observations point to a role for CD45 in regulating survival and apoptotic pathways in immune cells (23, 29–31).

With regard to HIV, although a role for CD45 has been proposed in HIV-infected cells (32, 33), its role in the bystander apoptotic effect of HIV on T cells has not been investigated. In this study, we therefore aimed to gain insight into the involvement of CD45 in the gp120-mediated apoptosis of uninfected bystander cells. Here, we analyzed the apoptotic responses and signaling pathways that are regulated by CD45 in mutant cell lines and peripheral blood mononuclear cells (PBMCs) using an in vitro model of HIV-1 envelope-mediated apoptosis. Our data show that CD45-negative cell lines (J45.01) are fairly resistant to gp120-induced apoptosis. Differential induction of Fas ligand (FasL) and the consequent activation of the Fas/FasL pathway were observed in CD45-positive and CD45-negative T cell clones upon gp120-induced apoptosis. We also found that CD45 regulates apoptosis via the phosphoinositide 3-kinase (PI3K)/Akt pathway. These findings implicate a novel role for CD45 in HIV-1 gp120-induced apoptosis in vitro.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—The human Jurkat T cell line (clone JE6.1) and the CD45-deficient variant of this clone (J45.01) were obtained from American Type Culture Collection (Manassas, VA). J45/CH11 (expressing a chimeric protein containing the extracellular and transmembrane domains of HLA-A2 and the phosphatase domain of CD45), its control J45/A2 (expressing the extracellular and transmembrane domains of HLA-A2), and J45/LB3 (J45.01 transfectants expressing normal human CD45) were kindly provided by Dr. Gary A. Koretzky (University of Pennsylvania School of Medicine) and Dr. Eric J. Brown (Uni-
_CD45 Regulates HIV-1 gp120-mediated Apoptosis_

versity of California, San Francisco, CA), respectively, and have been described previously (27, 36). All cell lines were cultured at 37 °C in 5% CO₂ in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 50 µg/ml penicillin, and 50 µg/ml streptomycin. Human PBMCs were isolated from heparinized blood samples obtained from normal healthy donors by Ficoll-Paque density gradient centrifugation (Amersham Biosciences). The mononuclear cell fraction was then depleted from macrophages by adherence to cell culture flasks for 1 h at 37 °C.

Antibodies to procaspase-3, Fas, Fasl, Lck, and paxillin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-Akt Ser473, anti-phospho-Lck Tyr505, anti-Akt, and anti-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-poly(ADP-ribose) polymerase antibody was obtained from Clontech. Anti-CD3 and anti-CD45 monoclonal antibodies were obtained from Immunotech and Pharmingen, respectively. Phyceroerythrin (PE)-labeled anti-CD4 antibody, PE-labeled anti-CXCR4 antibody, PE-labeled anti-mouse IgG, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Fab’2) (BD Biosciences) were used for the flow cytometric studies. Anti-gp120 (HIV-1) monoclonal antibody was obtained from ImmunoDiagnostics, Inc. (Woburn, MA).

_Reagents—Electrophoresis reagents and nitrocellulose membranes were obtained from Bio-Rad. The selective CD45 inhibitor (a pivalamide with a 9,10-phenanthrenedione core) was obtained from Calbiochem. The specific PI3K inhibitors wortmannin and LY294002, sodium orthovanadate, staurosporine, and the protease inhibitors were purchased from Sigma. HIV-1 gp120 (IIIB) was obtained from Protein Sciences Corp. (Meriden, CT).

_Cell-surface Staining and Flow Cytometric Analysis—To detect CD4 and CXCR4 expression, Jurkat cells were stained directly with specific PE-labeled antibodies. For CD3 and CD45 expression, the cells were stained with anti-CD45 and anti-CD3 primary monoclonal antibodies, respectively, followed by fluorescein isothiocyanate-conjugated secondary antibody. Flow cytometry was carried out using a FACS Calibur, and the data were analyzed using CellQuest software (BD Biosciences).

_Induction and Detection of Apoptosis—_Cells from various clones of the human Jurkat T cell line or mutant cell lines or PBMCs (2 × 10⁶/ml) were subjected to 10 nM recombinant HIV-1 gp120 (IIIB) for 30 min at 37 °C. They were then washed once, treated with an equal concentration of mouse anti-gp120 (HIV-1) monoclonal antibody (NIH AIDS Research and Reference Reagent Program, Germantown, MD), incubated at 4 °C for 30 min, washed once, and seeded in 12-well plates coated with anti-CD3 antibody (2 µg/ml in phosphate-buffered saline (PBS) for 1 h at 37 °C). Untreated cells and cells treated with anti-CD3 antibody alone were used as controls. Anti-CD3 antibody was used at a suboptimal concentration so that it would be unable to trigger significant apoptosis when used alone. Apoptosis was detected at the end of a 48-h incubation at 37 °C using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) kit (Roche Applied Science). Briefly, cells were fixed in freshly prepared paraformaldehyde solution (4% in PBS at pH 7.4) for 1 h, washed with PBS, incubated in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice, washed with PBS, and then incubated in the TUNEL reaction mixture at 37 °C in the dark. Cells were washed again three times with PBS before analysis by flow cytometry. The net percentage of apoptosis was calculated by subtracting the apoptosis in the presence of immobilized anti-CD3 antibody alone from the apoptosis observed upon treatment with gp120/anti-gp120 antibody/immobilized anti-CD3 antibody. For induction of staurosporine- and anti-Fas antibody-mediated apoptosis, the cells were treated with 100 nM staurosporine and 100 ng/ml agonistic anti-Fas monoclonal antibody CH11 (Calbiochem). For induction of apoptosis using γ-irradiation, cells were subjected to a dose of 10 grays/min at room temperature using an irradiator (CIS Biointernational, Bedford, MA). In addition, to generate anti-CD3 antibody-induced apoptosis, the cells were treated with 20 µg/ml immobilized anti-CD3 antibody coated onto 12-well plates as described above.

_Inhibition of gp120-induced Apoptosis—_For the inhibition experiments, cells (2 × 10⁶/ml) were pretreated with the indicated doses of PI3K inhibitors and the CD45 phosphatase inhibitor for 1 h at 37 °C and then subjected to apoptosis according to the protocol described above. The inhibitors were maintained throughout all passages of apoptosis induction at the same doses used for the pretreatment.

_Immunoblotting and Immunoprecipitation—_Aliquots of total protein extracts (50 µg) were run on 8% SDS-polyacrylamide gel (Invitrogen) and transferred to nitrocellulose membranes. Immunodetection involved specific primary antibodies, appropriate secondary antibodies conjugated to horseradish peroxidase, and the ECL chemiluminescence detection system (Amersham Biosciences). Equal loading and transfer were monitored by probing for anti-actin or anti-paxillin antibody.

For immunoprecipitation, equal amounts of protein from the stimulated time points were clarified by incubation with protein A-Sepharose CL-4B or GammaBind™ Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. The Sepharose beads were removed by brief centrifugation, and the supernatants were incubated with different primary antibodies for 2 h at 4 °C. Immunoprecipitation of the antigen/antibody complexes was performed by overnight incubation at 4 °C with 50 µl of protein A-Sepharose or GammaBind™-Sepharose (50% suspension). Non-specific interacting proteins were removed by washing the beads three times with radioimmune precipitation buffer and once with PBS. Immunoprecipitated complexes were solubilized in 50 µl of 2× Laemmli buffer and further analyzed by immunoblotting as described above.

_CD45 Activity in Cell Lysates—_CD45 tyrosine phosphatase activity in the lysates of Jurkat T cells was measured using the colorimetric human active CD45 DuoSet IC assay (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol. In brief, an immobilized antibody specific for CD45 was used to capture both active and inactive CD45. After washing away unbound material, a synthetic phosphopeptide substrate was added and was then dephosphorylated by active CD45 to generate free phosphate and unphosphorylated peptide. The free phosphate was detected by a sensitive dye binding assay using malachite green and molybdic acid at 620 nm on a microplate reader. CD45 tyrosine phosphatase activity was determined by calculating the rate of phosphate release.

_Small Interfering RNA (siRNA)-mediated Knockdown—_RNAi-mediated knockdown of CD45 was performed using synthetic duplex RNA oligonucleotides as described by Oliveira and Goodell (34). The 19-nucleotide targets for siRNAs were as follows: GUCUUUGUCA-CAGGGGCAA (Dharmacon, Inc., Boulder, CO). PBMCs were electroporated with 100 nM siRNA using the Amaxa system (Amaxa GmbH, Cologne, Germany). CD45 siRNA-mediated knockdown was estimated by detection of CD45 receptor expression 48 h after the initial transfection by flow cytometry.

_gp120 Binding Assay—_For determination of gp120 binding, cells (2 × 10⁶/ml) were treated with HIV-1 gp120 (10 nM) for 30 min at 37 °C, then with equal concentrations of anti-gp120 (HIV-1) antibody for 30 min at 4 °C, and finally with fluorescein-conjugated goat anti-mouse IgG F(ab’)2 (1:50 dilution) for 30 min at 4 °C. The samples were analyzed by flow cytometry.
**Transient Transfection**—The Akt constructs were kindly provided by Dr. Michael E. Greenberg (Harvard Medical School, Boston, MA). Plasmid carrying the hemagglutinin (HA)-tagged Akt derivative rendered kinase-inactive by mutation within the catalytic domain of Akt (HA-Akt(K179M)), plasmid carrying HA-tagged wild-type Akt, or the pcDNA control vector was transfected into Jurkat T cells by electroporation using the Amaxa Nucleofector system.

**Statistical Analysis**—The results are expressed as the means ± S.D. of data obtained from three or four experiments performed in duplicate or triplicate. Statistical significance was determined by Student’s t test.

**RESULTS**

In this study, we used a model in which HIV-1 gp120 cross-linked with anti-gp120 antibody induces apoptosis of Jurkat T cells and PBMCs in the presence of CD3/TCR activation. This model has been used previously in several studies involving gp120-induced apoptosis (12, 13, 35). In fact, this system is equivalent to the *in vivo* situation, in which gp120 can be found in HIV-1-infected individuals on the surface of infected cells, on the surface of free virus, or shed from infected cells. In addition, the host generates anti-gp120 antibody as an immune response to the virus. Hence, such gp120/anti-gp120 antibody complexes, bound to CD4 in the uninfected cells in the presence of an activating antigen or infectious agent, receive stimulatory signals through TCR, which may translate into increased levels of apoptosis.

**Differential Susceptibility of the CD45 Clones to gp120-induced Apoptosis**—We demonstrated the role of CD45 in gp120-induced apoptosis by examining J45.01, a CD45-deficient Jurkat cell line. This Jurkat cell variant expressed minimal amounts of CD45 on its surface, yet expressed other T cell-surface markers (including CD3, CXCR4, and CD4) at levels similar to those observed in the wild-type Jurkat cells (Fig. 1). The CD45-deficient J45.01 cells showed a reduced apoptotic response to gp120 (10 nM) in comparison with the control JE6.1 Jurkat cells (Fig. 2A). The difference in apoptotic response to gp120 was consistent over a concentration range of 10–100 nM (data not shown). We used TUNEL assays to evaluate apoptosis.

To determine whether the observed difference in apoptosis between the wild-type Jurkat cells and J45.01 cells was due to the lack of CD45 molecules, gp120-induced apoptosis was evaluated in J45.01 cells that were reconstituted with normal human CD45 (J45/LB3). The reconstituted cell line (J45/LB3) and the wild-type Jurkat cells (JE6.1) expressed comparable levels of CD45 (Fig. 2B, left panel). Susceptibility to gp120-induced apoptosis was restored to ~80% in the J45/LB3 cells (Fig. 2B, right panel).

**Knockdown of CD45 in Primary T Cells Leads to a Decrease in gp120-induced Apoptosis**—To further confirm, by an independent method, the effect of CD45 down-regulation on gp120-induced apoptosis of primary cells, we utilized siRNA-driven CD45 knockdown (34) in PBMCs. As shown in Fig. 2C (left panel), there was significant knockdown of CD45 in PBMCs transfected with the CD45 siRNA compared with the control (irrelevant) siRNA. When the cells transfected with the CD45 siRNA were subjected to gp120-induced apoptosis (Fig. 2C, right panel), there was an ~35% reduction in apoptosis, thus corroborating the results observed with the CD45-deficient cell line. The above data further substantiate the key role of CD45 in apoptosis induced by gp120.

**Caspase Activation Is Decreased in CD45-deficient Cells**—To quantify the levels of apoptosis, we monitored several established apoptotic markers,
FIGURE 2. CD45 is necessary for optimal gp120-mediated apoptosis. A, CD45-positive (JE6.1) and CD45-negative (J45.01) cells were treated with 10 nM gp120 and an equal concentration of anti-gp120 antibody and cultured with immobilized anti-CD3 antibody for 48 h. Controls included JE6.1 and J45.01 cells treated with anti-CD3 antibody, gp120, or anti-gp120 antibody alone. The cells were stained using the TUNEL assay as described under "Experimental Procedures," and the percentage of apoptosis was determined by flow cytometry. *, \( p < 0.05 \) versus wild-type Jurkat T cells (JE6.1). Data are the means ± S.D. of three independent experiments.

B, CD45-positive (JE6.1) and CD45-reconstituted (J45/LB3) cells were stained using anti-CD45 antibody (Ab) and then analyzed by flow cytometry (left panel). Cells stained with control IgG represent the antibody control. CD45-positive (JE6.1), CD45-negative (J45.01), and CD45-reconstituted (J45/LB3) cells were stimulated with gp120, and apoptosis induction was determined at 12, 24, and 48 h by TUNEL staining. The percentage of apoptosis was determined by flow cytometry (right panel). Data are the means ± S.D. of triplicate assays. FITC, fluorescein isothiocyanate.

C, control siRNA- and CD45 siRNA-transfected PBMCs were stained using anti-CD45 antibody and analyzed by flow cytometry (left panel). Cells stained with control IgG represent the antibody control. The histogram plot represents a comparison of mock-, control siRNA-, and CD45 siRNA-transfected cells subjected to gp120-induced apoptosis after 48 h of incubation (right panel). *, \( p < 0.05 \) versus the control siRNA sample. Data are the means ± S.D. of three independent experiments.

D, gp120-induced activation of caspase-3 (left panel) and cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase (right panel) were decreased in CD45-deficient cells (J45.01). Caspase activation was determined by Western blot analysis of the cytosolic extracts from J45.01 (CD45 \(^{-/-}\)) and JE6.1 (CD45 \(^{+/+}\)) cells 24–48 h after gp120 treatment. Equal loading and transfer were monitored by probing for anti-paxillin antibody. Results are representative of three independent experiments.
including detection of caspase-3 and cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase. Caspases are key regulators of the induction and execution of apoptosis, with caspase-3 being a crucial executor for the apoptotic process. As shown in Fig. 2D (left panel), there was decreased processing of procaspase-3 in the J45.01 cells (CD45^{+/+}) compared with the wild-type JE6.1 cells (CD45^{+/+}) upon treatment with gp120. We found that cleavage of poly(ADP-ribose) polymerase by caspase-3 was also reduced in the CD45-deficient cells (Fig. 2D, right panel).
CD45 Regulates HIV-1 gp120-mediated Apoptosis

The Extracellular Domain and the Intracellular Phosphatase Domain of CD45 Are Responsible for Optimal gp120-induced Apoptosis—CD45 is a transmembrane tyrosine phosphatase with an extracellular domain, a transmembrane domain, and an intracellular phosphatase domain. Here, we chose to examine the structural components of CD45 that are important for HIV gp120-induced apoptosis. For this purpose, we analyzed the ability of transfectants expressing hybrid CD45 cDNA to restore gp120-induced apoptosis (36). As shown in Fig. 3A, the CD45-deficient Jurkat cells expressing a chimeric protein consisting of the extracellular and transmembrane domains of the major histocompatibility complex class I protein and the cytoplasmic domain of CD45 (J45/CH11) were not able to restore the gp120-induced apoptosis. As a control, we used a CD45-deficient cell line with only the extracellular and transmembrane domains of the major histocompatibility complex class I protein (J45/A2). These results suggest the involvement of the extracellular domain of CD45 in regulating gp120-induced apoptosis.

In addition, we evaluated the effect of a recently discovered CD45 phosphatase inhibitor (37) on gp120-induced apoptosis. The inhibitor is a cell-permeable pivalamide with a 9,10-phenanthreneone core that has been shown to act as a potent, selective, and reversible inhibitor of CD45 phosphatase (37). We found that this selective CD45 inhibitor blocked gp120-induced apoptosis of both JE6.1 cells and PBMCs at a concentration of 100 nM (Fig. 3B). These results indicate that expression of full-length CD45 consisting of both the extracellular domain and the intracellular phosphatase domain may be necessary for gp120-mediated apoptosis.

CD45 Tyrosine Phosphatase Activity Is Modulated by HIV-1 gp120—We analyzed CD45 tyrosine phosphatase activity in Jurkat T cells upon stimulation with gp120 alone, CD3 ligation alone, or their combination at various apoptotic time points. Although CD3 ligation increased CD45 tyrosine phosphatase activity only slightly, gp120 alone or in combination with anti-CD3 antibody caused a significant increase in CD45 tyrosine phosphatase activity (Fig. 3C). This phenomenon was further confirmed by immunoblotting the lysates with anti-phospho-Lck Tyr505 antibody. The Tyr505 site on Lck is a direct substrate of CD45 tyrosine phosphatase (38). As shown in Fig. 3D, gp120 alone or in the presence of CD3 ligation caused significant dephosphorylation of Lck at Tyr505 at the apoptotic time points. Equal protein loading was monitored using anti-Lck antibody.

CD45 Does Not Regulate Other Major Apoptotic Pathways—The inability of J45.01 cells to undergo apoptotic cell death cannot be attributed to the lack of apoptotic machinery because these cells were triggered to undergo apoptosis by other apoptotic pathway inducers such as anti-Fas antibody, staurosporine, and γ-irradiation (Fig. 4, A–C, respectively). In addition, we also evaluated apoptosis in response to a higher concentration (20 μg/ml) of anti-CD3 antibody (Fig. 4D). The difference in apoptosis induced in the wild-type and CD45-deficient clones was not statistically significant.

CD45 Does Not Regulate gp120 Binding and gp120-induced Internalization of CD4—We ruled out several potential mechanisms for the anti-apoptotic effect of the J45.01 cells in response to gp120. No significant difference was detected in the ability of the CD45-positive and CD45-negative cells to bind gp120 at a concentration of 10 nM (Fig. 5A), the amount normally used to induce apoptosis. To determine whether the cell-surface expression of CD95 (Fas receptor), CD3, CXCR4, and CD4 was changed upon gp120 treatment, JE6.1 and J45.01 clones were analyzed by flow cytometry at various time points using the model of gp120 stimulation as described under “Experimental Procedures.” There was no change in the expression of CD95 or CD3 in either of the cell lines after gp120 treatment (data not shown). We observed only a...
moderate internalization of CXCR4 (20–25%) upon gp120 stimulation. However, no difference in the gp120-induced internalization of CXCR4 was observed between the CD45-positive and CD45-negative cells (data not shown). In the case of the CD4 receptor, although there was marked down-regulation of CD4 upon gp120 treatment in the JE6.1 and J45.01 cells, there was no statistical difference between the two cell lines (Fig. 5B). These results suggest that the apoptotic function of CD45 is attributable to defective signaling rather than to the modified expression of these molecules.

Cross-talk between CD45 and the CXCR4 Receptor upon gp120 Stimulation—To further investigate the role of T cell receptor components in HIV-1 gp120-induced apoptosis, we analyzed the cross-talk between CXCR4 and the TCR complex-mediated signaling pathway. Cell lysates obtained from CD45-positive JE6.1 cells or peripheral blood lymphocytes stimulated with gp120 were immunoprecipitated with anti-CXCR4 antibody and subjected to Western blot analysis with anti-CD45 antibody. We found that the gp120/ci-mouse antibody treatment of JE6.1 cells (Fig. 6A) and PBMCs (Fig. 6B) in the presence of CD3 ligation induced CXCR4 receptor association with CD45. Equal amounts of protein were present in each lane as detected by immunoblotting of the lysates with anti-actin antibody (Fig. 6, A and B, lower panels).

CD45 Regulates the Induction of FasL during gp120-induced Apoptosis—We next sought to determine the CD45-regulated pathways that may have a role in inducing gp120-mediated apoptosis. gp120-mediated apoptosis has been shown to occur upon induction of FasL and the subsequent triggering of the Fas (CD95) pathway (13, 35). Because CD45-positive and CD45-negative clones have similar levels of Fas expression (data not shown) and are both susceptible to Fas-mediated apoptosis (Fig. 4A), the above observations suggest the possibility that differential induction of FasL may be responsible for the varied sensitivity of the clones to gp120-induced apoptosis. To test this hypothesis, we analyzed FasL protein expression as well as Fas receptor expression after gp120 stimulation at various time points. As shown in Fig. 7, the induction of FasL expression by gp120 was inhibited in the CD45-deficient J45.01 cells compared with the wild-type JE6.1 cells, whereas there were no differences observed in Fas expression between the two cell lines. Equal protein loading was monitored using anti-paxillin antibody. These findings indicate that, by failing to up-regulate FasL in response to gp120, the J45.01 cells may become resistant to apoptosis.

CD45 Regulates the PI3K/Akt Pathway—Growing evidence indicates that the PI3K/Akt pathway is an important regulator of FasL expression (39). This pathway has been shown to regulate survival signals that protect cells from apoptosis in multiple cell lines (40–42). Hence, we evaluated the phosphorylation of Akt at Ser473 in both JE6.1 and J45.01 cells upon gp120 stimulation. We observed an enhanced phosphorylation of Akt upon CD3 stimulation, which was substantially inhibited by HIV-1 gp120 treatment in the CD45-positive cell line. However, gp120 did not inhibit the phosphorylation of Akt in the CD45-deficient J45.01 cells (Fig. 8A, left panel). The phosphorylation indices for Akt are shown in Fig. 8A, right panel).

The Wild-type Active Form of Akt Partially Prevents gp120-induced Apoptosis—To further confirm the role of Akt, we transfected an HA-tagged wild-type Akt- or mutant Akt(K179M)-expressing construct or the pcDNA3 vector alone into Jurkat (JE6.1) cells. Western blot analysis with anti-HA antibody revealed the expression of HA in the transfectants (Fig. 8B). There was increased phosphorylation of Akt at Ser473 in the wild-type Akt-overexpressing cells compared with the Akt(K179M)-overexpressing cells, which showed decreased phosphorylation of Akt at Ser473 (Fig. 8B). Equal protein loading was monitored using anti-actin antibody.

We then analyzed the effect of overexpressing wild-type Akt or Akt(K179M) on gp120-induced apoptosis. We observed an ~50% reduction in gp120-induced apoptosis of the wild-type Akt-transfected cells compared with the pcDNA control vectortransfected cells (Fig. 8A).
**CD45 Regulates HIV-1 gp120-mediated Apoptosis**

**A.**

|            | Anti-CD3 | Gp120/anti-gp120 |
|------------|----------|------------------|
| Time (min) |          |                  |
| C          | -        | -                |
| 0.5        | +        | +                |
| 2.5        | +        | +                |
| 5          | -        | -                |
| 15         | +        | +                |

**JE6.1**

IP: CXCR4; WB: CD45

**CD45**

Actin

**B.**

|            | Anti-CD3 | Gp120/anti-gp120 |
|------------|----------|------------------|
| Time (min) |          |                  |
| C          | -        | -                |
| 0.5        | +        | +                |
| 2.5        | +        | +                |
| 5          | -        | -                |
| 15         | +        | +                |

**PBMCs**

IP: CXCR4; WB: CD45

**CD45**

Actin

**DISCUSSION**

Indirect mechanisms of T cell loss as a consequence of immune activation have been proposed to play a key role in HIV pathogenesis (14). Cell death resulting from exacerbation or inappropriate induction of activation-induced apoptosis represents an important mechanism that leads to depletion of uninfected bystander CD4+ T cells in HIV-infected patients (14, 15, 45). Previous studies have shown that CD4+ T cells undergo apoptosis following HIV-1 gp120/CD4 cross-linking in the presence of CD3/TCR activation (12, 13, 35). In our study, we used the above in vitro model because it mimics the in vivo situation, in which circulating gp120 as well as membrane-associated gp120 can bind to receptors on uninfected cells, leading to apoptosis (12). Recent studies on activation-induced apoptosis have suggested that some of the TCR proximal signals leading to the death of activated T cells are similar to those leading to proliferation and differentiation (46, 47). Given the critical role of CD45 in regulating proximal signaling events in T cells, we explored here the role of CD45 in the HIV-1 envelope-induced apoptosis of T cells. Although it has been shown that CD45 phosphatase activity modulates HIV infection in T cells (32, 33), our data provide the first demonstration of the prominent role played by CD45 in the gp120-induced apoptosis of bystander T cells. Several lines of evidence support this conclusion.

Growing evidence suggests that CD45 may function in the regulation of apoptosis. CD45 has been implicated in the death of both thymocytes and PBMCs (23, 31). CD45-null thymocytes from CD45-deficient mice are severely impaired in their apoptotic response upon TCR engagement (23). In vitro studies have shown that CD45 is required for the monocyte-dependent apoptosis of human T cells (31) as well as galectin-1-induced cell death (30). In our studies using mutant CD45 cell lines, we found that the CD45-deficient cell lines were significantly resistant to gp120-induced apoptosis, as shown in our in vitro model. The role of CD45 was confirmed in primary T cells using a selective CD45 phosphatase inhibitor and siRNA. To exclude the role of CD45 in the other major apoptotic pathways, we subjected CD45-deficient cells to inducers of other apoptotic pathways. CD45-negative cells behaved similarly to the wild-type Jurkat cells in their susceptibility to apoptosis induced by anti-CD3 antibody, anti-Fas antibody, staurosporine, and...
γ-irradiation, indicating that CD45 has no significant role to play in these apoptotic pathways. This finding is consistent with a previously published observation suggesting that CD45 is not required for Fas receptor-mediated death signals (48). The ability of a higher concentration of anti-CD3 antibody to induce similar levels of apoptosis in the CD45-positive and CD45-negative cells also underscores the specificity of the role of CD45 in gp120-induced apoptosis in the presence of low doses of anti-CD3 antibody.

In our study, the incomplete inhibition of gp120-induced apoptosis of the CD45-negative cells indicates that the pathway mediated by CD45 may not be the only one responsible for gp120-induced apoptosis. Our findings are in agreement with those of Westendorp et al. (13), who showed that the gp120/anti-gp120 antibody-mediated apoptosis of Jurkat T cells was partially inhibited by reagents that block the binding of FasL to the Fas receptor. An alternative pathway for apoptosis induction by gp120 in CD45-negative cells has in fact been proposed by Berndt et al. (49), who showed the rapid Fas-independent death of CD4+/CD4- T cells mediated through CD4 and CXCR4. The induced cell death was independent of known caspases and lacked oligonucleosomal DNA fragmentation.

The membrane-bound tyrosine phosphatase CD45 consists of an extracellular domain, a transmembrane domain, and a cytoplasmic tail that possesses phosphatase activity. CD45 exists in multiple isoforms because of the various ectodomains formed due to alternative splicing of these exons (designated A, B, and C) (50). The function of the intracellular phosphatase activity of CD45 has been well established because it regulates primarily T cell activation via TCR (20). However, the role of the extracellular domain of CD45 in its function is not well known. In this study, we examined the structural components of CD45 that are important for HIV-1 gp120-induced apoptosis. We found that CD45-negative transfectants expressing a chimeric protein consisting of the extracellular and transmembrane domains of the major histocompatibility complex class I protein (HLA-A2) along with the cytoplasmic domain of CD45 (36) failed to undergo gp120-induced apoptosis. Furthermore, we have shown that a selective inhibitor of CD45 phosphatase activity also significantly inhibited gp120-induced apoptosis. These
data clearly indicate that both the extracellular and phosphatase domains of CD45 are important for HIV-1 gp120-induced apoptosis. Our study complements a previous study in which the extracellular domain of CD45 was shown to be important for apoptosis induced by galectin-1 (51). The involvement of the extracellular domain could indicate that one or more of the protein partners that co-associate with CD45 via its extracellular domain on the T cell surface are required for transmitting the apoptotic signal. We were able to show an increase in the CD45 protein-tyrosine phosphatase activity of T cells upon treatment with HIV-1 gp120 at various apoptotic time points. Currently, little is known about the mechanisms by which CD45 activity is regulated. However, it is possible that HIV-1 gp120 induces CD4/p56ɛκ modifications, which may in turn affect CD45 function. In addition, we were able to show a gp120-induced interaction of CD45 and CXCR4 in both the Jurkat cell line and PBMCs. During T cell activation, CD45 positions itself in close proximity to the CD3/CD4 receptors and is physically and functionally associated with these molecules, leading to its activation (50). Also, CD45 has been shown to associate with CXCR4 upon gp120 stimulation. We hypothesize that, in the presence of CD3/TCR activation, gp120 may interfere with the CD45 and CD3/CD4 association and also cause an interaction between CD45 and CXCR4 either directly or indirectly. The HIV-1 gp120-induced association of CXCR4 with CD45 may regulate substrate access to the tyrosine phosphatase domain by targeting CD45 to distinct areas in the membrane, where it can modulate the function of molecules important for apoptotic signaling by altering their phosphorylation status and activity. This could lead to deleterious TCR signaling, in turn leading to apoptosis.

We further investigated the molecular mechanism by which CD45 regulates apoptosis. FasL is a critical activator of the activation-induced apoptosis of mature T cells (52, 53). Previous studies have suggested an important role for Fas/FasL interactions in HIV pathogenesis (54–56). Furthermore, increased levels of soluble and cell-associated FasL expression have been reported in the lymphocytes of HIV-1-infected patients, correlating with the HIV RNA burden (57, 58). Moreover, it has been shown that, like activation-induced apoptosis, gp120-induced apoptosis occurs in T cells via Fas/FasL interactions (13, 35). We have shown that overexpression of wild-type CD45 Regulates HIV-1 gp120-mediated Apoptosis

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