HIV-1 envelope induces activation of caspase-3 and cleavage of focal adhesion kinase in primary human CD4$^+$ T cells

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Binding of HIV type 1 (HIV-1) envelope glycoproteins to the surface of a CD4$^+$ T cell transduces intracellular signals through the primary envelope receptor, CD4, and a coreceptor, either CCR5 or CXCR4. Furthermore, envelope–CD4$^+$ cell interactions increase rates of apoptosis in peripheral blood mononuclear cells (PBMCs). We demonstrate that in primary T lymphocytes, recombinant HIV-1 envelope proteins induce the activation of caspase-3 and caspase-6, which belong to a family of cysteine proteases that, upon activation, promote programmed cell death. Envelope-mediated activation of caspase-3 and caspase-6 depended on envelope–CD4 receptor interactions; CCR5-utilizing as well as CXCR4-utilizing envelopes elicited this response. Focal adhesion kinase (FAK) is a substrate of both caspase-3 and caspase-6, and inactivation of FAK by these caspases promotes apoptosis. Envelope treatment of lymphocytes led to the cleavage of FAK in a manner consistent with caspase-mediated cleavage.

The depletion of CD4$^+$ T cells is a central pathogenic feature of HIV type 1 (HIV-1) infection and is largely responsible for the profound immunodeficiency that is characteristic of late stages of HIV disease. However, in seropositive individuals, only a very small proportion of CD4$^+$ T cells are productively infected at any given time (1). This observation has given rise to a great deal of interest in other mechanisms whereby HIV-1 might induce the depletion of CD4$^+$ cells, including CD4$^+$ T cells that are not infected (2–5). One of the principal mediators of immune system dysfunction is the virally encoded envelope protein gp120/41, either presented on the surface of infected cells, on viral particles, or as a soluble protein (6, 7). The presence of high concentrations of HIV envelope in anatomic sites important to disease pathogenesis (i.e., lymphoid tissues) underscores the potential for envelope to contribute to T cell dysfunction (7). HIV envelope treatment of PBMCs results in the induction of apoptosis, both in CD4$^+$ and CD8$^+$ T lymphocytes (8, 9). The increase in apoptosis results directly from the interaction of envelope with CD4$^+$ cells, as well as indirectly through the expression and/or secretion of factors capable of inducing apoptosis (8). The precise mechanism(s) by which HIV envelope induces apoptosis in lymphocytes is controversial; both Fas-dependent (10–12), and Fas-independent (8, 13, 14) mechanisms have been reported. It is clear, however, that not all lymphocytes are equally susceptible to envelope-mediated apoptosis (3, 15).

A number of proteins responsible for transmitting apoptotic signals within cells have recently been identified. External apoptotic stimuli, as well as certain intracellular events, result in the activation of signal transduction pathways that converge on a cascade of proteases known as caspases (16, 17), which execute the process of programmed cell death. Caspases comprise a family of cysteine proteases that cleave at the C terminus of aspartic acid. They are all expressed as 30- to 50-kDa proenzymes that require proteolytic processing for activation (6, 7, 17–19). Selected sets of protein substrates are cleaved upon activation by individual caspases in a coordinated manner (6, 7, 17–19). In most instances, this cleavage results in a loss or change of function (6, 7, 17–19). A prominent role of effector caspases is to inactivate proteins that protect living cells from apoptosis. Of note, caspases have been implicated in HIV-mediated apoptosis (20). In addition, caspase inhibitors suppress HIV envelope-mediated apoptosis, providing indirect evidence that the envelope glycoprotein may itself activate caspases (21). Finally, patients with progressive HIV disease demonstrate increased caspase-3 activity (22).

We have previously demonstrated that HIV envelope treatment of CD4$^+$ T cells results in the phosphorylation of focal adhesion kinase (FAK) and its recruitment into focal adhesion complexes (23). In addition, we and others have shown that envelope treatment of CD4$^+$ cells leads to the phosphorylation of Pyk2 (23, 24). In certain cell types, phosphorylated FAK provides a protective effect against apoptosis, while the phosphorylation of PYK2 is associated with increased levels of apoptosis (25). Because HIV envelope is known to induce apoptosis in peripheral blood mononuclear cells (PBMCs), we sought to determine whether envelope treatment of PBMCs might subsequently lead to the inactivation of FAK. Cleavage of FAK by caspase-3 and caspase-6 has been reported, and leads to the disassembly of focal adhesion complexes, an event that promotes programmed cell death (26–29). Therefore, we determined whether HIV envelope induces the activation of caspase-3 and caspase-6 in CD4$^+$ and CD8$^+$ T lymphocytes, and whether this activation of caspases might be associated with cleavage of phosphorylated FAK.

Materials and Methods

Cells and Reagents. For caspase activation assays, terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL), and Annexin V staining, PBMCs were freshly isolated from individuals by Ficoll/Hypaque centrifugation. Cells from normal donors were determined to be homozygous for the wild-type CCR5 allele by PCR as previously described (30). Cells were cultured in RPMI 1640 medium/10% FBS. For FAK immunoblot analysis, cells were obtained by leukapheresis, followed by rosetting with neuraminidase-treated sheep RBC. CD4$^+$ T cells were purified from the rosette-positive T cell-enriched fraction as previously described (23).

Antibodies used for immunoprecipitation, flow cytometry, and Western blot analysis were used according to the manufacturers' recommendations and included anti-phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY), anti-

Abbreviations: FAK, focal adhesion kinase; PBMC, peripheral blood mononuclear cell; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; sCD4, soluble CD4; FasL, Fas ligand; PE, phycoerythrin; mcf, mean channel fluorescence.

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pyk2 (clone 11; Transduction Laboratories, Lexington, KY), anti-FAK (clone 77 monoclonal, Transduction Laboratories; C903 polyclonal, Santa Cruz Biotechnology; A-17 polyclonal, Santa Cruz Biotechnology), and phycoerythrin (PE)-conjugated anti-active caspase-3 (PharMingen).

All envelope proteins have been expressed and purified as previously described (30, 31), and are available through the AIDS Reference and Reagent Program (http://www.aidsreagent.org). Envelopes utilized included those derived from HIV-1 JRFL, a CCR5-utilizing molecular clone (33); HIV-1 NL43, a tissue culture adapted CXCR4-utilizing molecular clone (34); HIV-1 92Ug20.9, a clade D primary CXCR4-utilizing biological clone (35); HIV-1 92MW959, a CCR5-utilizing biological clone (35); and SIV PBj, a CCR5/STRL33-utilizing molecular clone (36).

Soluble CD4 (sCD4) (SmithKline Beecham) was used to interfere with the interaction between HIV-1 envelope and CD4. The anti-Fas mAb CH11 (Beckman Coulter) was used as a positive control to induce Fas-mediated apoptosis. Recombinant Fas ligand (FasL) (Alexis, San Diego, CA) was also used as a positive control in apoptosis assays.

Immunoprecipitation and Western Blot Analysis. $CD4^+$ T cells (60×10^6) isolated as described above were lysed in a buffer containing 50 mM Tris·HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 μM β-glycerophosphate, and 10 mM sodium fluoride. The protein concentration of the lysates was determined by using a colorimetric assay according to the manufacturer's instructions (Bio-Rad). Proteins were immunoprecipitated with Protein A agarose-conjugated anti-phosphotyrosine antibody (Upstate Biotechnology) overnight at 4°C with constant rotation. Immunoprecipitates were washed four times in lysis buffer, then resuspended in Laemmli sample buffer and subjected to SDS/PAGE on 8–16% polyacrylamide gradient gels. Proteins in these gels were transferred to Immobilon-P membranes (Millipore). Membranes were blocked with 5% BSA in TBS containing 0.1% Tween 20 (TBS-T), followed by incubation with primary antibody at 1 μg/ml overnight at 4°C. After further washing in TBS-T, membranes were incubated with a 1:10,000 dilution of secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia) in 5% BSA/TBS-T. Signal detection was achieved with an enhanced chemiluminescence kit (Amersham Pharmacia) and Biomax MR film (Eastman Kodak).

Caspase-3 Immunostaining Assay. Caspase-3 proenzyme is cleaved into an active form that is preferentially recognized by a rabbit anti-active caspase-3 polyclonal antiserum (PharMingen). Freshly isolated PBMCs (1×10^6) were treated with 5–20 nM HIV envelope or an equivalent concentration of anti-Fas mAb CH11 or FasL (0.5 μg/ml) for approximately 5 h at 37°C and subsequently permeabilized with Cytofix/Cytoperm permeabilization reagent (PharMingen). Permeabilized cells were stained with PE-conjugated anti-active caspase-3, and analyzed on a Becton Dickinson FACScan flow cytometer.

Caspase-3 and Caspase-6 Activity Assay. CellProbe DEVD-Rho and VEID-Rho (Beckman Coulter) are peptide substrates that are hydrolyzed by caspase-3 and caspase-6, respectively, to form a nonfluorescent leaving group and fluorescent Rhodamine-110, which can be measured by flow cytometric analysis. Freshly isolated PBMCs (5×10^5) were treated with 5–20 nM HIV envelope or an equivalent concentration of anti-Fas mAb CH11 for approximately 5 h at 37°C. Fluorescence detection was carried out according to the manufacturer's instructions.

Annexin V Staining. Freshly isolated PBMCs (5×10^5) were cultured in RPMI 1640 medium/10% FBS in 96-well plates. Cells were treated with 5–20 nM HIV envelope for approximately 16 h at 37°C and subsequently stained with Annexin V FITC (R & D Systems) and propidium iodide per the manufacturer's instructions.

TUNEL Assays. Freshly isolated PBMCs (2×10^5 cells) were cultured in RPMI 1640 medium/10% FBS in 96-well plates. Cells were treated with 5–20 nM HIV envelope for approximately 56 h at 37°C, treated with terminal deoxynucleotidyltransferase, and stained with biotin UTP followed by streptavidin-PE according to the manufacturer's instructions (Beckman Coulter).

Results

HIV Envelope Proteins Induce the Activation of Caspase-3 in PBMCs. Caspase-3 is expressed as a 32-kDa proenzyme, which is activated by proteolytic cleavage into an active 21/17-kDa form. To determine whether HIV envelope induces the activation of

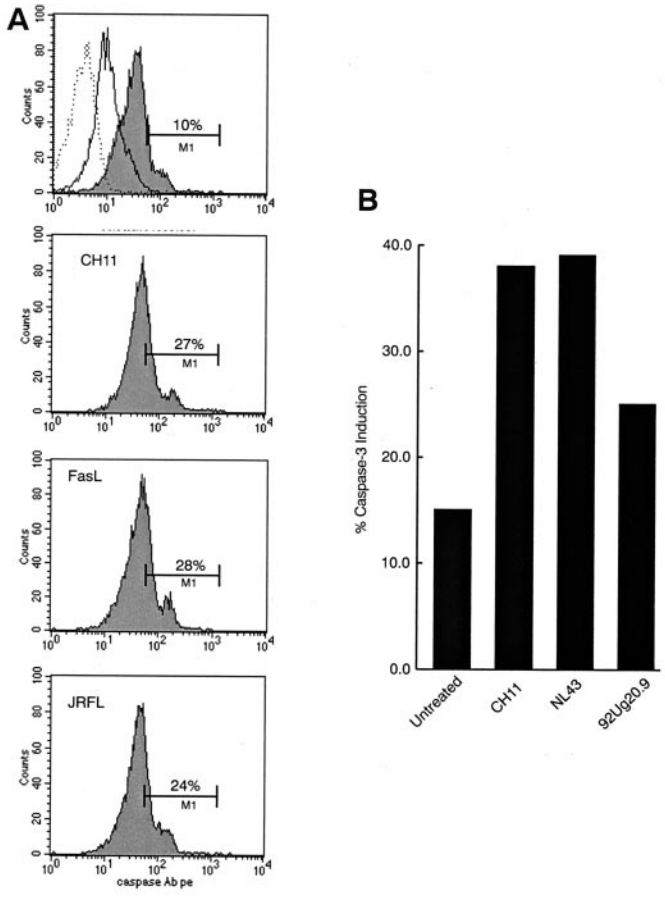

Fig. 1. Induction of caspase-3 activation determined by immunostaining. Flow cytometric analyses of PBMCs untreated or treated with anti-Fas mAb CH11, FasL, or HIV-1 JRFL envelope are shown in *A*. Additional results are presented in *B* with HIV-1 NL43 and 92Ug20.9 envelopes and with anti-Fas mAb CH11. In the histograms in *A*, mean channel fluorescence (mcf) is displayed on the *x* axis and total cell counts on the *y* axis; the gate was set on the $CD3^+$ population. The dotted line represents unstained cells, the solid line represents the isotype control, and the shaded areas show anti-active caspase-3 staining. The marker boundary (M1) was set to exclude background staining in the untreated cells (*Top*); the percentage of cells with mcf values above the marker boundary is indicated in each histogram. (*B*) mcf values over background are presented as determined for *A*.

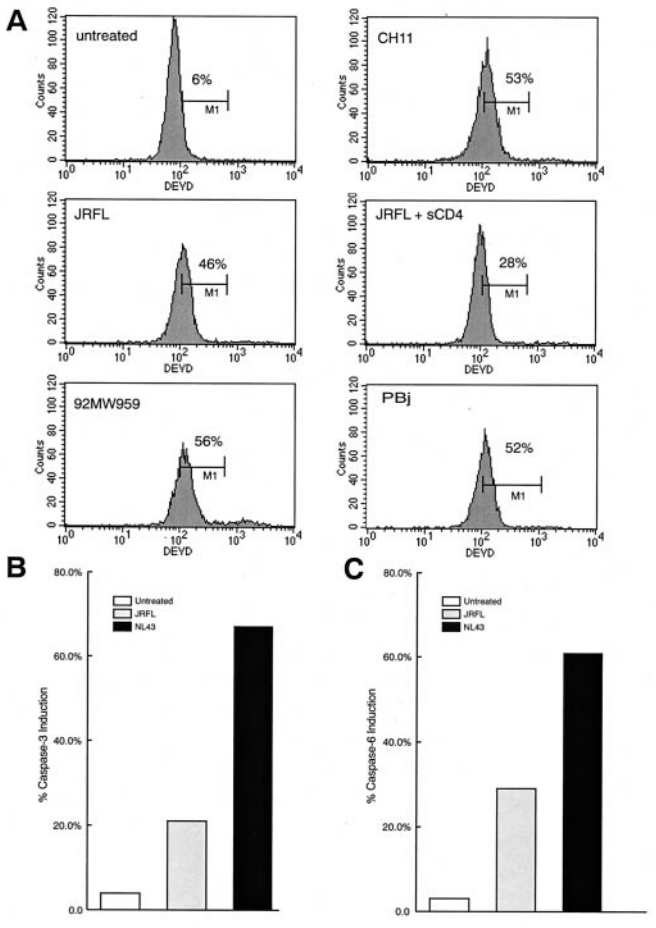

Fig. 2. Activation of caspase-3 and caspase-6 by HIV and SIV envelopes. Flow cytometric analyses of PBMCs treated with various recombinant envelopes or the anti-Fas mAb CH11, in the presence of a fluorogenic tetrapeptide substrate for caspase-3 (DEVD-Rho; *A* and *B*), or caspase-6 (VEID-Rho; *C*) are shown. In the histograms, mcf is displayed on the *x* axis and total cell counts on the *y* axis; cells were gated on the $CD3^+$ population. The anti-Fas mAb CH11 was employed as a positive control. The marker boundary (M1) was set to exclude background staining in the untreated cells; the percentage of cells with mcf values above the marker boundary is indicated in each histogram. (*B* and *C*) mcf values over background are presented as in *A*.

caspase-3 in $CD4^+$ and $CD8^+$ T lymphocytes, we employed an anti-active caspase-3 polyclonal antiserum that preferentially recognizes the cleaved/activated form of caspase-3 (37). Freshly isolated PBMCs were treated with recombinant HIV-1 JRFL, NL43, or 92Ug20.9 envelope proteins. Induction of caspase-3 activation in $CD3^+$ T cells was observed as early as 5 h, and up to 24 h after treatment with all three recombinant proteins (Fig. 1). Treatment of cells with either FasL or the anti-Fas mAb CH11 resulted in a degree of caspase-3 induction that was comparable to that induced by HIV envelope proteins (Fig. 1).

To verify the results obtained above, we employed a second caspase-3 assay based on the enzymatic cleavage of the fluorogenic substrate, DEVD-rhodamine (DEVD-Rho). In addition, we employed a second fluorogenic substrate, VEID-rhodamine (VEID-Rho), that is specifically cleaved by caspase-6. Upon cleavage, these rhodamine-conjugated substrates fluoresce in the range of 504–541 nanometers, which can be detected by flow cytometric analysis. Soluble recombinant HIV-1 JRFL, NL43, and 92MW959 and SIV PBj envelope proteins were tested for their ability to induce the cleavage of these substrates in freshly isolated PBMCs. Activation of caspase-3 in $CD3^+$ T cells was

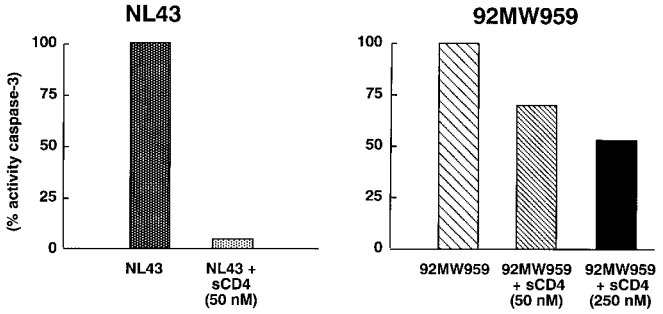

Fig. 3. Inhibition of envelope-induced activation of caspase-3 by sCD4. Fresh PBMCs were treated with HIV envelopes NL43 or 92MW959 for 5 h in the presence or absence of sCD4, with the addition of the caspase-3 substrate DEVD-Rho in the last hour of incubation. Cells were then analyzed by flow cytometry. The values presented indicate the percent mcf of cells treated with envelope minus the percent mcf of untreated cells, with the response to envelope set at 100%. Cells were gated on the $CD3^+$ population.

observed within 5 h after treatment with all four recombinant envelope proteins as well as with the anti-Fas mAb CH11 (Fig. 2). Caspase-6 was activated by three of the four envelope proteins (Fig. 2*C* and data not shown). Of the envelope proteins that were assayed, the one derived from NL43 induced the greatest degree of caspase-3 activation in both the caspase-3 enzymatic assay as well as the active-caspase-3 immunostaining assay (Figs. 1 and 2).

HIV Envelope-Mediated Caspase-3 and Caspase-6 Activation Is Dependent on CD4 Receptor–Envelope Interactions. Both CCR5- and CXCR4-utilizing envelopes were capable of mediating caspase-3 and caspase-6 activation, suggesting that this phenomenon was either mediated through CD4, either of the HIV coreceptors, or CD4 in combination with a coreceptor. To discriminate between these possibilities, we examined the capacity of sCD4 to inhibit or enhance envelope-mediated caspase activation. sCD4 would be expected to inhibit a process that was entirely CD4 receptor-dependent, but might enhance a process that depended on interactions between CD4 and a coreceptor (32). As shown in Fig. 3, sCD4 strongly inhibited NL43 envelope-mediated caspase-3 activation, indicating that this phenomenon is mediated through the CD4 receptor. Activation of caspase-3 in PBMC treated with 92MW959 envelope (Fig. 3) or JRFL envelope (Fig. 2) was only partially inhibited by sCD4. The differential effect of sCD4 on NL43 envelope vs. 92MW959 or JRFL envelopes is consistent with the differential neutralizing properties of sCD4 for primary and tissue culture-adapted isolates (38, 39). To further investigate the role of coreceptors in caspase activation, we examined PBMCs derived from an individual who was homozygous for an inactivating 32-bp deletion in the CCR5 gene (CCR5-Δ32) (40, 41). Caspase-3 and caspase-6 were induced by JRFL envelope in PBMCs derived from a CCR5-Δ32 donor (Fig. 4). Taken together, these results suggest that CD4 receptor engagement by envelope provides a sufficient stimulus for caspase-3 and caspase-6 activation. This is consistent with previous reports which demonstrate that CD4 crosslinking by anti-CD4 mAbs provides the necessary stimulus to induce apoptosis directly in $CD4^+$ and indirectly in $CD8^+$ T lymphocytes (42, 43). However, these results do not exclude additional roles for CCR5 or CXCR4.

Previous reports have demonstrated that envelope mediates apoptosis in both $CD4^+$ and $CD8^+$ subsets of T lymphocytes (8, 44–47). Both soluble factors (46), and cell surface receptors (8), have been identified as mediators of these effects. Subset analysis of envelope-treated cells indicated that caspase-3 was activated in both $CD4^+$ and $CD8^+$ T cell subsets (data not

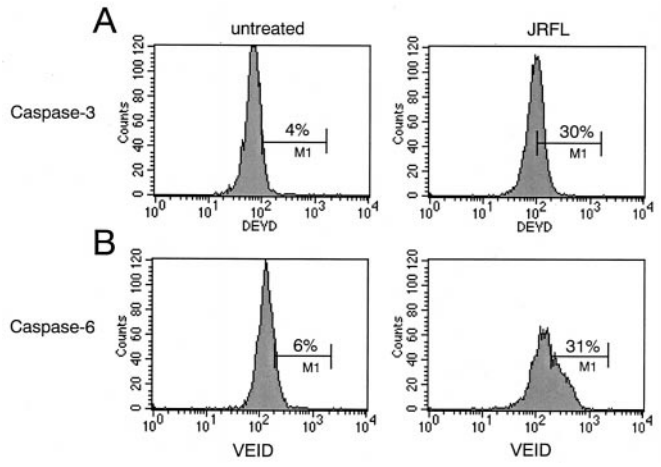

Fig. 4. Caspase-3 and caspase-6 activation in response to HIV envelope proteins in a donor homozygous for CCR5-Δ32. Flow cytometric analyses are shown of CCR5-Δ32 donor PBMCs treated with JRFL envelope for 5 h, with the addition of the caspase-3 substrate DEVD-Rho (*A*) or the caspase-6 substrate VEID-Rho (*B*) in the last hour of incubation. mcf is displayed on the *x* axis and total cell counts on the *y* axis. Cells were gated on the $CD3^+$ population. The marker boundary (M1) was set to exclude background staining in the untreated cells; the percentage of cells with mcf values above the marker boundary is indicated in each histogram.

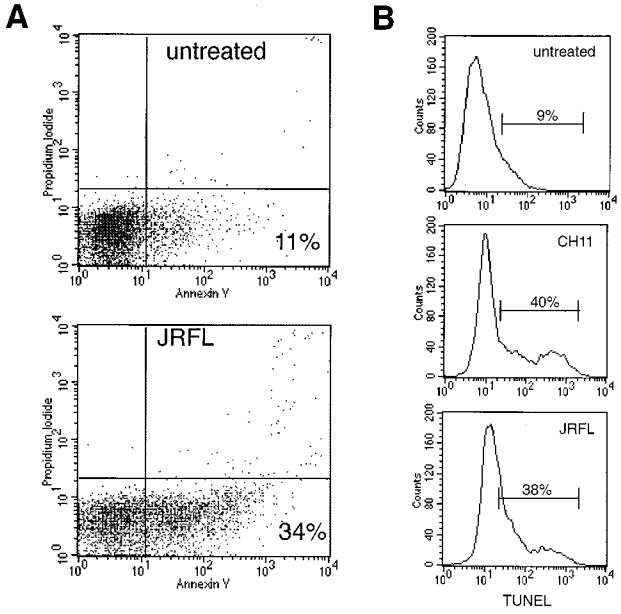

Fig. 5. Induction of apoptosis by HIV-1 envelope proteins. Annexin V staining and TUNEL assay of PBMCs. Annexin V and propidium iodide staining of PBMCs treated with HIV-1 JRFL envelope for 16 h is shown in *A*. The percentage of Annexin V-positive/propidium iodide-negative cells is indicated in the lower right quadrant of the histograms in *A*. TUNEL assay of PBMCs untreated or treated with anti-Fas mAb CH11 or HIV-1 JRFL envelope for 56 h is shown in *B*. mcf is displayed on the *x* axis and total cell counts on the *y* axis. The percentage of TUNEL-positive cells is presented for each panel.

shown). Because envelope-mediated caspase activation was CD4 receptor-dependent (Fig. 3), we conclude that activation of caspases in CD4-negative cells likely occurs through indirect mechanisms (8, 44–47).

Envelope-Induced Caspase-3 Activation Correlates with Envelope-Induced Apoptosis. It has been reported that the activation of caspase-3 in lymphocytes reflects a commitment to apoptosis (19). To determine whether the observed caspase-3 activation correlated with other well-characterized markers of apoptosis, we analyzed envelope-treated cells for annexin V content by immunostaining and for DNA fragmentation by the TUNEL assay. Annexin V-positive cells increased from 11% to 34% of the total PBMC population 16 h post envelope treatment (Fig. 5). When these same cells were assayed at 56 h for the presence of cleaved chromosomal DNA, the percentage of TUNEL-positive cells increased 4-fold from 9% to 38%. These increases are similar in degree to those obtained from cells staining positive for the active form of caspase-3 (Fig. 1), demonstrating a good correlation between envelope-induced caspase-3 activation and envelope-induced apoptosis.

FAK Is Cleaved in a Caspase-Specific Pattern in T Lymphocytes Stimulated with HIV Envelope. We previously demonstrated that HIV envelope induces the tyrosine-phosphorylation of FAK (23). Because activated FAK is a substrate for both caspase-3 and caspase-6 (26–29), we asked whether phosphorylated FAK was subject to caspase-3 and caspase-6 cleavage upon treatment of $CD4^+$ T cells with HIV envelope. Several different caspase-3/caspase-6 cleavage products of FAK have been identified; among those best characterized are two fragments of 36 kDa and 32 kDa (26–29). After treatment of cells with HIV envelope, immunoprecipitation with anti-phosphotyrosine antibody and immunoblot analysis with an anti-FAK polyclonal antiserum revealed the presence of these 36- and 32-kDa FAK cleavage products (Fig. 6). In some experiments, we also observed a ≈75-kDa FAK fragment (Fig. 6*A*), consistent with observations reported by van de Water *et al.* (28). In contrast to FAK, the closely related tyrosine-kinase PYK2, which is also phosphorylated upon envelope treatment, remained intact (Fig. 6*B*). Despite the appearance of FAK cleavage products, full-length FAK was still detected (Fig. 6*A*). This may indicate that distinct subsets of $CD4^+$ T cells respond differentially to envelope treatment. We conclude that envelope treatment of $CD4^+$ T cells leads to the phosphorylation of FAK and to the activation of caspase-3 and

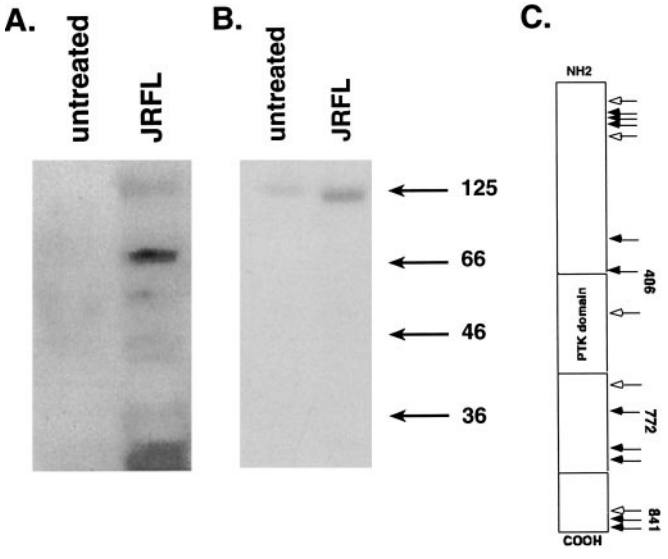

Fig. 6. HIV envelope-induced FAK fragments consistent with cleavage by caspase-3 and caspase-6. An anti-FAK Western blot of untreated or HIV-1 JRFL envelope-treated $CD4^+$ T cell lysates (6×10^7 cells each lane) followed by immunoprecipitation with an anti-phosphotyrosine mAb is shown in *A*. (*B*) Immunoprecipitates were stained with anti-PYK2 antiserum. (*C*) A schematic of all reported caspase-3 and caspase-6 cleavage sites within FAK.

caspase-6. The phosphorylated form of FAK, which is a substrate of these caspases, is cleaved in a manner consistent with proteolysis by caspase-3 and caspase-6 (Fig. 6*C*).

Discussion

The immunodeficiency that defines AIDS results from a progressive decline in the number of $CD4^+$ T lymphocytes. Marked impairment in the functional capability of $CD4^+$ T lymphocytes as well as in other cellular immune responses additionally characterizes HIV disease (48, 49). These deficiencies in immune function are associated with the replication of HIV and/or the production of HIV-encoded gene products (50–60). Although no single viral protein is likely to be the sole effector of immune dysfunction, the viral envelope is believed to contribute significantly to immune system impairment (reviewed in refs. 61 and 62). Effects of HIV envelope proteins on $CD4^+$ T cells include aberrant signal transduction (23, 24, 63–65), anergy (63, 64, 66, 67), inappropriate trafficking (65), and apoptosis (5, 43). In this study, we demonstrated that HIV envelope proteins induce the activation of caspase-3 and caspase-6 in a CD4 receptor-dependent manner. A principal function of these two proteases is the cleavage of substrates, which in their cleaved forms promote apoptosis (16, 17). The activation of these proteases is likely the molecular mechanism responsible for the known apoptotic activity of HIV envelope. HIV envelope proteins activated caspases in both $CD4^+$ and $CD8^+$ T cells; inhibition of this activation by sCD4 in both cellular subsets indicates that the effect of HIV envelope proteins on $CD8^+$ T cells is indirect, consistent with previous observations (8, 43–46).

We have reported previously that HIV envelope induces the formation of an activation complex that includes phosphorylated FAK and the HIV coreceptor CCR5 (23). We postulated that the formation of this complex may contribute to the dysregulation of cellular activation and trafficking associated with HIV infection. In addition, we noted that these complexes may facilitate HIV entry. In the present study, we have demonstrated that the phosphorylated form of FAK can be cleaved in a manner consistent with caspase-3 and/or caspase-6 proteolysis. This proteolysis may play a significant role in envelope-mediated apoptosis. Cleavage of FAK by caspase-3 is associated with the early stages of apoptosis (25–29). Thus, the activation and subsequent cleavage of phosphorylated FAK may represent critical early steps in HIV-driven programmed cell death. We previously reported that envelope activates FAK in a subset of T cells (23). It remains to be determined whether those cells in which FAK is phosphorylated and then cleaved by caspases defines the subset of lymphocytes susceptible to envelope-mediated apoptosis. In addition, we speculate that cells in which FAK is activated but not cleaved defines a subset of $CD4^+$ T cells that escape envelope-mediated apoptosis.

In conclusion, the present study demonstrates that HIV envelope proteins induce the activation of caspase-3 and caspase-6 in lymphocytes in a CD4 receptor-dependent process, and that this is likely an important mechanism in HIV envelope-mediated apoptosis of T lymphocytes. HIV envelope also induces the activation of FAK, which is cleaved in a caspase-3/caspase-6 specific pattern. These events further elucidate the potential mechanisms that lead to the loss of $CD4^+$ T cells and the impairment of immune function in HIV-infected individuals.

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