Requirement of Calmodulin Binding by HIV-1 gp160 for Enhanced FAS-mediated Apoptosis

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Accelerated apoptosis is one mechanism proposed for the loss of CD4+ T-lymphocytes in human immunodeficiency virus type 1 (HIV-1) infection. The HIV-1 envelope glycoprotein, gp160, contains two C-terminal calmodulin-binding domains. Expression of gp160 in Jurkat T-cells results in increased sensitivity to FAS- and ceramide-mediated apoptosis. The pro-apoptotic effect of gp160 expression is blocked by two calmodulin antagonists, tamoxifen and trifluoperazine. This enhanced apoptosis in response to FAS antibody or Cε-ceramide is associated with activation of caspase 3, a critical mediator of apoptosis. A point mutation in the C-terminal calmodulin-binding domain of gp160 (alanine 835 to tryptophan, A835W) eliminates gp160-dependent enhanced FAS-mediated apoptosis in transiently transfected cells, as well as in vitro calmodulin binding to a peptide corresponding to the C-terminal calmodulin-binding domain of gp160. Stable Tet-off Jurkat cell lines were developed that inducibly express wild type gp160 or gp160A835W. Increasing expression of wild type gp160, but not gp160A835W, correlates with increased calmodulin levels, increased apoptosis, and caspase 3 activation in response to anti-FAS treatment. The data indicate that gp160-enhanced apoptosis is dependent upon calmodulin up-regulation, involves the activation of caspase 3, and requires calmodulin binding to the C-terminal binding domain of gp160.

HIV-1 infection is characterized by immune system hyperactivation and dysfunction that increases with disease progression until immune function is lost. During the long asymptomatic phase, a state of equilibrium appears to be achieved, consisting of a rapid turnover of lymphocytes concomitant with rapid viral production and clearance (1–3). This equilibrium eventually shifts with a loss of the ability of the immune system to replace CD4+ cells and a gradual increase in viral load. Since HIV-1 primarily infects CD4+ T-lymphocytes, and the decline in these cells along with the rise in viral load is a hallmark of disease progression, understanding the relationship between these phenomena is critical in understanding HIV-1 pathogenesis.

One proposed mechanism for the accelerated loss of CD4+ cells is an increased rate of apoptosis (4). Apoptosis, or programmed cell death, differs from necrosis in that dying cells participate actively in their own death and generally do not induce an inflammatory response (5). Physiologically, apoptosis functions in maintaining tissue homeostasis and is important in removal of lymphocytes after an immune response. The latter function prevents accumulation of lymphocytes in the blood and is mediated by interaction of the apoptotic receptor, FAS, with FAS ligand. Upon binding FAS ligand, FAS recruits signaling molecules to the death-inducing signaling complex (6–8). Induction of this pathway leads to activation of caspase 3, subsequent activation of caspase 6, and eventually activation of DNA fragmentation factor and a Ca2+/Mg2+-dependent endonuclease responsible for cleavage of DNA resulting in the typical laddering pattern seen in most forms of apoptosis (9–11).

The importance of apoptosis in AIDS is controversial although there is abundant in vitro evidence supporting a role for apoptosis in the pathogenesis of HIV-1 (12), and although mechanisms regulating this apoptosis in vivo are not clear, current evidence implicates the FAS pathway. Evidence supporting this hypothesis includes reports that lymphocytes from HIV-1-infected individuals express elevated levels of FAS and are more sensitive to FAS-mediated apoptosis in vitro compared with non-infected individuals (13–15). The chronically activated immune state in HIV infection may lead to an improperly elevated rate of apoptosis in vivo. Additionally, the level of spontaneous apoptosis in lymphocytes from HIV+ individuals is elevated and positively correlates with increasing disease severity (12, 16).

The coat glycoprotein of HIV-1, gp160, is post-translationally cleaved to an extracellular subunit, gp120, and a transmembrane subunit, gp41. The subunits are non-covalently associated, and both are required for viral entry into the cell. Two calmodulin-binding sites have been identified near the C terminus of gp160 (17). Purified gp160 or peptides corresponding to the gp160 calmodulin-binding sites bind calmodulin in vitro and inhibit calmodulin-dependent enzymes (18). Expression of gp160 in Molt4 cells, a FAS-resistant human T-cell line, rendered these cells sensitive to FAS-induced apoptosis (19), whereas this effect was diminished in Molt4 cells expressing gp160Δ147, a truncated glycoprotein that lacks both calmodulin-binding domains (19). Further evidence implicating calmodulin in the cytopathic effect of gp160 is that two calmodulin antagonists, tamoxifen and trifluoperazine, block FAS-mediated apoptosis in gp160-expressing cells (19). In addition, tamoxifen and trifluoperazine have been reported to reduce spon-
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taneous apoptosis in cultured peripheral blood mononuclear cells from HIV-1-infected individuals (16).

We demonstrate here that gp160 expression enhances FAS-mediated apoptosis in Jurkat cells by increasing calmodulin expression and accelerating caspase 3 activation and that these effects that require calmodulin binding to gp160 are blocked by a single point mutation in the C-terminal calmodulin-binding domain.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Jurkat cells were purchased from the ATCC (Manassas, VA) and grown at 37 °C in 5% CO2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

**Plasmids**—Plasmid pSRHS containing the HIV-1 envelope gene, gp160, and the truncated forms of gp160, Δ147, which lacks the C-terminal 147 amino acids of gp41, and Δ67, which lacks the C-terminal 67 amino acids of gp41, as well as the plasmid pKS8, containing the cDNA for β-galactosidase under control of the human β-actin promoter, were kindly provided by Eric Hunter, Department of Microbiology, University of Alabama at Birmingham, and were used in all transfection experiments.

**Transfections**—All transient transfections were performed using the cationic lipid, DMRIE-C (Life Technologies, Inc.). Briefly, lipid-DNA complexes were allowed to form for 45 min at room temperature in serum-free medium. Cells were added to the complex in serum-free medium and incubated for 5 h at 37 °C in 5% CO2. RPMI 1640 medium (Life Technologies, Inc.) was added, and cells were cultured an additional 48 h. Transfection efficiency is monitored by simultaneous transfection of the β-galactosidase expression vector, pKS8. Efficiencies ranged from 50 to 90% in Jurkat cells.

**Antibodies and Reagents**—Monoclonal antibody to calmodulin was developed as described previously (20) and is available from Upstate Biotechnology Inc. Lake Placid, NY. Mouse anti-human FAS monoclonal antibody (Upstate Biotechnology Inc.) was used to induce apoptosis. Anti-human caspase 3 monoclonal antibody was from Transduction Laboratories (San Diego, CA), caspase 3 polyclonal antibody was from PharMingen (San Diego, CA); C6-ceramide and C6-dihydrorademamide was from Avanti Polar Lipids (Birmingham, AL).

**Site-directed Mutagenesis**—Stratagene’s (La Jolla, CA) QuikChange Site-directed Mutagenesis Kit was used to make point mutations of gp160 according to manufacturer’s instructions. Primers for the mutagenic sites were purchased from Life Technologies, Inc. and correspond to the desired mutation of alanine 835 to tryptophan flanked by 10–15 base pairs of correct sequence, using the sequence of HIV-1 strain HXB2 as a template.

**Creation of gp160- and gp160A835W-expressing Cell Lines**—Tet-Off Jurkat cells (CLONTECH, Palo Alto, CA) were transfected with the pTRC99A vector containing gp160 A835W under control of tetracycline by electroporation. Selection of stable cell lines was initiated 48 h after transfection using 100 μg/ml geneticin, 300 μg/ml hygromycin, and 2 μg/ml tetracycline in RPMI 1640 medium and incubated complete medium changed every 4 days. After 5–7 days, living cells were separated from dead cells and plated at a lower density. After serial dilution, isolated single cell clones were cultured in 96-well plates and then transferred into 12-well and 6-well plates and a 25-cm2 T flask.

**Northern Blot of gp160 and gp160A835W in Tet-off Jurkat Clones Induced by Removal of Tetracycline**—Jurkat clones expressing HIV-1 gp160 and mutant gp160A835W were incubated with decreasing doses of tetracycline for 48 h. Total RNA was isolated by the guanidinium thiocyanate/phenol/chloroform method and separated by formaldehyde gel electrophoresis. RNA was blotted onto Hybond N membranes and prehybridized for 45 min at 68 °C and then hybridized for 1 h using Quikhyb (Stratagene). The hybridization probe was generated by random labeling with Prime-a-Gene Labeling System (Promega, Madison, WI) with [α-32P]dCTP and HIV-1 HXB2 envelope cDNA template.

**Peptide Synthesis**—Peptides corresponding to the C-terminal calmodulin-binding site of gp160 (residues 826–843) were synthesized at the University of Alabama at Birmingham Comprehensive Cancer Center Peptide Synthesis and Analysis Shared Facility and purified by high pressure liquid chromatography. Wild type gp160 peptide sequence is 826-DRIVEVQGACRAIRHIP-843, and gp160A835W sequence is 826-DRIVEVQGWCRAIRHIP-843, with the residue corresponding to 835 underlined.

**TdT Apoptosis Staining**—In situ apoptosis staining was performed using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals). Briefly, cells were collected after treatments and cytopsin onto microscope slides and fixed in 10% formalin in PBS. Slides were then rinsed and treated with 20 μg/ml Proteinase K (Fisher) for 15 min at room temperature. Slides were washed several times with water and incubated with at 37 °C TdT (0.3 units/ml), digoxigenin-labeled dUTP, and buffer (30 μM Tris base, pH 7.5, 140 mM sodium cacodylate, 1 μM cobalt chloride). After a brief wash, slides were blocked in 1% bovine serum albumin, 0.1% gelatin in PBS for 15 min at room temperature. Slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody for 1 h at room temperature, washed in PBS, and developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphosphate (Sigma) at room temperature for 30 min. Slides were coveredslipped and at least 400 cells per slide were counted to determine the percentage of apoptotic cells.

**Western Blot for Caspase 3 and Calmodulin**—Jurkat cells were treated as indicated and lysed in buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM sodium ortovanadate, 10 mM EDTA, 10 mM EGTA, 1 mM ammonium molybdate, 50 mM NaF, 0.5 μM okadaic acid, 5 mM benzamidine, and 50 μg/ml pepstatin. Equivalent amounts of protein (100 μg) were separated on 12.5% SDS-PAGE and transferred to Immobilon P membrane (Millipore, Bedford, MA). Membranes were incubated in 0.2% glutaraldehyde in Tris-buffered saline (TBS) for calmodulin only, blocked in TBS containing 2% bovine serum albumin and 0.2% gelatin, and then incubated with a monoclonal caspase 3 antibody diluted 1:1000, a polyclonal caspase 3 antibody (1:5000), or a monoclonal calmodulin antibody (1:300 dilution) in TBSTween 20 (TBS) and anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech), 1:5000 in TTBS followed by development with enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Dansyl-Calmodulin Binding**—The binding of dansyl-calmodulin to peptides corresponding to the C-terminal calmodulin-binding domain of gp160 or the A835W mutant was determined fluorimetrically with dansyl-calmodulin (Sigma, St. Louis, MO) as reported previously (21). Briefly, fluorescence emission of dansyl-calmodulin was scanned from 400 to 600 nm after excitation at 340 nm with the indicated concentrations of peptide. All assays were performed in buffer containing 50 mM MOPS, pH 7.3, 200 mM KC1, and 1 μM CaCl2 with or without 5 mM EGTA. Emission spectra were obtained with concentrations of peptides ranging from 0 to 1 μM, and mellitin was used as a positive control for binding.

**RESULTS**

**Enhancement of Ceramide- and FAS-mediated Apoptosis by gp160**—Jurkat cells were transfected with gp160 or mock-transfected with empty vector and treated with a monoclonal FAS antibody (clone CH11), and apoptosis was determined as described under “Experimental Procedures.” Similar levels of apoptosis (11% in mock-transfected and 13% in gp160-transfected) were observed in cells not treated with FAS antibody (Fig. 1A). Cells transfected with gp160 undergo significantly more apoptosis (35 ± 3%) than vector-transfected cells (25 ± 1%) after treatment with FAS antibody (n = 8, p < 0.01). There was no difference in basal or FAS-mediated apoptosis between vector-transfected cells and untransfected Jurkat cells (data not shown).

Previous studies have shown that ceramide levels increase in HIV-1-infected cells in vitro (22) and that treatment of latently infected cells with ceramide induces viral production (23). Furthermore, ceramide is a reported second messenger in FAS-mediated apoptosis. To delineate the site of action of gp160, we tested the effect of gp160 on ceramide-mediated apoptosis in Jurkat cells. The specificity of ceramide action was demonstrated using dihydroceramide as a negative control (Fig. 1B). Dihydroceramide is a biologically inactive molecule, which differs from ceramide by one double bond (24). The ability of gp160 to enhance ceramide-mediated apoptosis is virtually identical to the effect of gp160 on FAS-mediated apoptosis (Fig. 1A). Ceramide treatment induced apoptosis in 32 ± 3% of gp160-transfected cells compared with 22 ± 3% in vector-transfected cells (n = 8, p < 0.001). Basal levels of apoptosis (13 ± 2% in gp160-transfected cells and 11 ± 2% in vector
Effect of Calmodulin Antagonists on gp160-enhanced Ceramide- and FAS-mediated Apoptosis—Jurkat cells were transfected with gp160 as described above and pretreated with 10 μM tamoxifen (TMX) or trifluoperazine (TFP) for 30 min prior to addition of FAS antibody (Fig. 2). Although TMX is widely used as an anti-estrogen, it is as potent a calmodulin antagonist as TFP in the range of 1–10 μM (25). TMX (n = 3) and TFP (n = 5) inhibited the FAS-mediated apoptosis in gp160-transfected cells by 75 ± 10 and 90 ± 2%, respectively (Fig. 2A).

The effects of TMX and TFP on gp160 enhanced ceramide-induced apoptosis were also tested to determine whether gp160-enhanced ceramide-mediated apoptosis is affected by calmodulin antagonists in a way that is similar to FAS-mediated apoptosis. Both TMX (n = 3) and TFP (n = 8) inhibited gp160-enhanced ceramide-mediated apoptosis by 65 ± 10 and 90 ± 5%, respectively (Fig. 2B). The data clearly indicate that the percent inhibition observed in these experiments is similar to the effect of TFP and TMX on FAS-mediated apoptosis in gp160-transfected cells.

Effect of gp160 Expression on Caspase 3 Activation by FAS and Ceramide—Since gp160 enhanced both FAS- and ceramide-mediated apoptosis, we investigated potential downstream mechanisms regulating this effect. Caspase 3 is recognized as an important effector molecule in the apoptotic pathway, and we hypothesized that gp160 expression may alter the activity of this protease. Caspase 3, like all known caspases, is synthesized as a zymogen and undergoes proteolytic activation in two steps. Cleavage by an upstream caspase followed by a second autocatalytic cleavage forms the fully active dimer of approximately 17 and 12 kDa. Enzyme activation in these studies is monitored by Western analysis for the 32-kDa proform (CPP32), which decreases upon enzyme activation. Cells treated with either FAS antibody or C2-ceramide for the indicated times demonstrate that the rate of caspase 3 activation is increased in cells transfected with gp160 as determined by Western blot analysis of Jurkat cell lysates from cells transfected with empty vector or vector containing gp160 (Fig. 3). The accelerated rate of decrease in the amount of the 32-kDa proform of the enzyme in response to both FAS antibody (Fig. 3A) and C2-ceramide (Fig. 3B) is evident. Thus, gp160 enhances both FAS- and ceramide-mediated apoptosis by a common mechanism involving activation of caspase 3.

Determination of the Amino Acid Residues of gp160 Critical to Calmodulin Binding and Enhanced Apoptosis—To test further the hypothesis that gp160-enhanced apoptosis requires calmodulin binding, we computer-modeled the three-dimensional structure of calmodulin bound to a peptide corresponding to the native C-terminal calmodulin-binding domain. The C-terminal rather than the N-terminal calmodulin-binding domain was selected because FAS-mediated apoptosis was not enhanced in cells transfected with wild-type gp160 (data not shown). Previous studies with this mutant determined that it also eliminated viral production in vitro (26). Computer modeling aligning the calmodulin-binding domain of myosin light chain kinase (MLCK) with the C terminus of gp160 identified five amino acids in the C-terminal calmodulin-binding domain of gp160 likely to be important for calmodulin binding (Fig. 4A, in bold). Calcium-bound calmodulin (Fig. 4B) has two globular domains, each containing two calcium-binding sites, separated by a highly flexible amphipathic helical region. It is this helical region that undergoes a major conformational change when calmodulin binds to target proteins by wrapping around the amphipathic helix of the target protein and bringing the two globular domains into close approximation.

An alanine 835 mutation to tryptophan in the gp160 peptide is predicted to be a substitution that will maximally affect...
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 binding to calmodulin because three-dimensional computer modeling predicts that this alanine fits in a tight hydrophobic pocket of calmodulin. Substitution of tryptophan for alanine is not predicted to change the overall conformation of gp160 but is predicted to disrupt binding to calmodulin, because the large side chain would be within 2 Å of five hydrophobic residues on calmodulin, too close to allow binding. The ribbon structure of calmodulin (calcium saturated) is illustrated in the absence of peptide (Fig. 4B) and in its predicted conformation when bound to wild type gp160 peptide (Fig. 4C). Ala-835 of gp160 is shown in purple and the five residues of calmodulin (Leu-18, Phe-19, Val-35, Leu-39, and Leu-112) predicted to be within 2 Å of the Trp residue of the mutant gp160 peptide are in orange.

Calmodulin Binding by gp160 Peptides—The effect of the A835W mutation of gp160 on calmodulin binding was tested directly using dansyl-calmodulin (dansyl-CaM) (Fig. 5). Dansyl-CaM is a fluorescently tagged calmodulin that binds target proteins with high affinity and has characteristic fluorescence emission spectra when excited at 340 nm, which increases upon binding to either calcium or peptides. Dansyl-calmodulin has previously been shown to activate calmodulin-dependent processes normally, indicating that the labeling has no effect on the biological activity of calmodulin (21). By titrating dansyl-CaM with increasing concentrations of peptide, dissociation constants for the dansyl-CaM-peptide complex may be obtained. Peptides corresponding to the C-terminal calmodulin-binding domain (wild type or A835W) were tested for their ability to bind dansyl-CaM, both in the presence and absence of calcium. Dansyl-CaM (100 nM) was titrated with increasing concentrations of either the wild type gp160 or A835W (Fig. 5B) peptides. Studies were performed in the presence of 1 mM CaCl2, and binding was shown to be calcium-dependent by chelating Ca2+ upon addition of 5 mM EGTA, which eliminated binding (data not shown). Emission spectra were obtained using dansyl-CaM alone or dansyl-CaM plus 5 μM wild type peptide (Fig. 5A) or 5 μM A835W mutant peptide (Fig. 5B). Only the wild type peptide significantly increased the fluorescence intensity of dansyl-CaM over the wavelengths scanned. The lack of increased emission intensity with the A835W peptide indicates that it does not bind to dansyl-calmodulin. A binding curve was generated by subtracting the fluorescence intensity of dansyl-CaM at 485 nm from the fluorescence intensity of dansyl-CaM plus peptide for each concentration of peptide as described previously (21). A representative curve shows saturable binding of gp160 peptide to dansyl-CaM (Fig. 5C).

Effect of gp160A835W on FAS-mediated Apoptosis—The effect of the A835W mutation on gp160-enhanced FAS-mediated apoptosis was determined by TdT staining of Jurkat cells treated with FAS antibody for 3 h. Transfection of the gp160 mutant, A835W, eliminated the enhancement of FAS-mediated apoptosis observed with wild type gp160 (Fig. 6). Apoptosis levels in gp160A835W-transfected cells were significantly reduced from wild type gp160-transfected cells (p = 0.009) and were nearly identical to levels of apoptosis measured in vector-transfected cells.

Tet-off Jurkat Cells Expressing gp160 or gp160A835W—To determine more precisely the effect of the A835W mutation of gp160 on FAS-mediated apoptosis, Jurkat Tet-off cell lines were created (as described under “Experimental Procedures”) that stably express either wild type gp160 or gp160A835W under tetracycline control. The tetracycline concentration-dependent expression of both glycoproteins was measured at the mRNA level after 48-h incubation with the indicated concentrations of tetracycline (Fig. 7). Representative Northern blots for wild type gp160 and gp160A835W demonstrate an increase in mRNA expression for both genes in response to decreasing tetracycline concentrations (Fig. 7A). Similar patterns of protein expression were also observed following removal of tetracycline and immunoprecipitating with HIV-infected human serum indicating that both glycoproteins were expressed at similar concentrations (data not shown).

Since earlier studies had shown an increase in calmodulin levels in cells transfected with gp160 but not a truncated form of gp160 (27), we tested whether a single point mutation affecting only one calmodulin-binding domain would be sufficient to eliminate increased expression of calmodulin. Tet-off Jurkat cells transfected with gp160 or gp160A835W were incubated with decreasing concentrations of tetracycline for 48 h. Cells were washed and lysed, and equivalent amounts of protein were separated by SDS-PAGE and Western-blotted for calmodulin (Fig. 7B). Calmodulin expression inversely correlates with tetracycline concentration in wild type gp160 cells but not in cells expressing gp160A835W. The pattern of calmodulin expression mirrors expression of wild type gp160 seen in Fig. 7A, with an approximate 2–3-fold increase in calmodulin expression in cells with the highest expression of gp160 (no tetracycline).

FAS-mediated Apoptosis and Caspase Activation in Tet-off gp160 and A835W Cells—FAS-mediated apoptosis was tested in the Tet-off cell lines, both in the presence of tetracycline and 48 h after removal of tetracycline from the culture medium (Fig. 8). In the presence of tetracycline, when glycoprotein expression is repressed, both wild type gp160 and gp160A835W-transfected lines respond to FAS identically, with approximately 20–30% of total cells being apoptotic (Fig. 8A). This level of apoptosis is consistent with the effect of anti-FAS treatment on mock-transfected Jurkat cells (Fig. 1A). In cells treated with anti-FAS for 3 h following removal of tetracycline, 70% of wild type gp160-expressing cells undergo apoptosis, an increase of 2.5-fold over uninduced cells, whereas the gp160A835W-expressing cells show no significant increase in apoptosis above levels seen in uninduced cells.

Parallel effects on caspase 3 activation were observed. Tet-off Jurkat cells expressing gp160 or gp160A835W were treated with anti-FAS for the indicated times, washed, and lysed, and equivalent protein was resolved by SDS-PAGE and Western-blotted for caspase 3 using an antibody that recognizes the active subunits of the enzyme (Fig. 8B). In these experiments, an increase in the active subunits on Western blot indicates activation of caspase 3. As expected, there is a substantial
increase in the amount of active subunits of caspase 3 in cells expressing wild type gp160. The presence of active caspase 3 is dramatically reduced in lysates from gp160A835W-expressing cells compared with lysates from wild type gp160-expressing cells. Active caspase 3 can be detected as little as 1 h following anti-FAS treatment of wild type-expressing cells, whereas very little active caspase 3 is seen in the gp160A835W-expressing cells even after 4 h of anti-FAS treatment.
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APPROACH AND EXPERIMENTAL PROCEDURES

RESULTS

Apoptosis in Jurkat cells transiently transfected with wild type gp160 and the A835W point mutant of gp160. Jurkat cells were transfected with empty vector, pSRHS, or vector containing wild type gp160, or the mutant gp160A835W. Cells were treated with 500 ng/ml anti-FAS antibody for 3 h and then TdT-stained. Results show the mean percentage apoptotic cells ± S.E. of four separate experiments. Cells transfected with gp160 underwent significantly more apoptosis in response to anti-FAS than did either vector-transfected (p = 0.002) or gp160A835W-transfected cells (p = 0.009).

Expression of gp160, gp160A835W, and calmodulin in Tet-off Jurkat cells. Tet-off Jurkat cells transfected with gp160 or gp160A835W were incubated for 48 h in decreasing concentrations of tetracycline (2 to 0 µg/ml), and expression of gp160 or gp160A835W mRNA was measured by Northern blot (A) as described under “Experimental Procedures,” or equivalent amounts of cell lysate were separated by SDS-PAGE and Western-blotted for calmodulin (B). Data are representative Western blots from each cell line with calmodulin indicated as CaM.

DISCUSSION

Research on HIV-1 and AIDS has recently led to development of treatments that have significantly decreased mortality in the United States. However, the current highly active antiretroviral treatment does not eliminate the virus from the body (28) and is far too expensive and complicated to be useful in developing nations where HIV infection is most prevalent. Therefore, understanding the interactions between a virus and its host remains essential for developing new therapeutic modalities. The molecular mechanisms responsible for the decline of CD4+ lymphocytes are unknown, although there is evidence that both FAS and FAS ligand expression are increased in AIDS patients (13, 29, 30), implicating FAS-mediated apoptosis as a potentially important mechanism.

Earlier work has shown that expression of HIV-1 envelope glycoprotein, gp160, overcomes a block in the FAS pathway in Molt4 cells (19), a FAS-resistant human T-cell line deficient in hematopoietic stem cell phosphatase. The results presented here support these earlier data and show that the effects of gp160 are not cell line-specific. These new data further support the hypothesis that calmodulin binding by gp160 is biologically relevant. Truncation mutants have suggested this, both in the work by Pan et al. (19) and by others (26) who showed that progressive reductions in the length of the cytoplasmic tail of gp160 had increasingly large effects on viral production in vitro.

The long cytoplasmic tail of gp160 is a feature conserved among HIV-1, HIV-2, and simian immunodeficiency virus. No specific function has been attributed to the cytoplasmic region, and despite considerable variability in the overall sequence, all known sequence variants of HIV-1 maintain the calmodulin-binding function (31). Cells transfected with gp160 are more sensitive to FAS-mediated apoptosis than mock-transfected cells, and this enhancement can be blocked by pretreatment with calmodulin antagonists (Fig. 2). The enhanced apoptosis is reduced in cells transfected with truncations of gp160 that remove most of the cytoplasmic tail of gp160, including the C-terminal calmodulin-binding domain (19).

To determine whether the mechanism of gp160-dependent enhanced apoptosis occurs at the level of the FAS receptor, we investigated whether transfection of gp160 enhanced ceramide-mediated apoptosis. Ceramide is a proposed mediator of apoptosis produced by cleavage of sphingomyelin by a cytosolic sphingomyelinase downstream of FADD in the FAS pathway (32). Whether ceramide generation is necessary for FAS-mediated apoptosis remains controversial (33–35), and we show that expression of gp160 enhances apoptosis induced by addition of a cell-permeable, synthetic ceramide compared with mock-transfected Jurkat cells. Calmodulin antagonists block gp160-enhanced ceramide-mediated apoptosis, suggesting that they function downstream of ceramidase.

Caspase 3, or caspase 3-like proteases, are critical effectors that play an important role in most types of apoptosis (8). During activation, caspase 3 is first cleaved by an upstream caspase (caspase 8 in the FAS pathway) and then undergoes a second autocatalytic cleavage to form the fully active protease. Cells transfected with gp160 and treated with FAS antibody or
ceramide activate caspase 3 more rapidly than mock-transfected Jurkat cells. The data suggest that the effects of gp160 are upstream of caspase 3 activation. This supports a previous report demonstrating that HIV infection-induced apoptosis could be blocked by caspase inhibition (36). Similarly, TMX and TFP block gp160-enhanced FAS- and ceramide-induced apoptosis, suggesting a calmodulin-dependent mechanism. The molecular site of action of calmodulin antagonists in gp160-enhanced apoptosis is currently under investigation. However, the data presented here indicate that calmodulin binding to gp160 is an early and required step in gp160-enhanced FAS- and ceramide-mediated apoptosis.

To confirm the effect of calmodulin binding by gp160 on FAS-mediated apoptosis, we created a point mutation in the C-terminal CaM-binding domain based on comparisons with the calmodulin-binding domain of MLCK (37–38). The sequence of the calmodulin-binding domains of both smooth muscle and skeletal muscle MLCK were compared with the gp160 calmodulin-binding sequence. However, the smooth muscle MLCK was used for computer modeling because its complex with calmodulin has been determined by crystal structure analysis (39). An alanine to tryptophan mutation at amino acid 835 of the gp160 peptide was created that was predicted not to change the overall amphipathic helical structure of the region but would replace a small hydrophobic amino acid with a much larger hydrophobic amino acid. The A835W mutant of gp160 failed to enhance FAS-mediated apoptosis above control levels (Fig. 7), indicating that this residue is critical to gp160 function and further supports the hypothesis that the interaction between gp160 and calmodulin is necessary to enhance apoptosis. Peptides corresponding to the C-terminal binding domain of the wild type gp160 and the A835W mutant were used in binding experiments with dansyl-calmodulin and demonstrated that only the wild type gp160 peptide bound dansyl-calmodulin. Binding of calmodulin by the wild type peptide was Ca2+-dependent (data not shown), and the affinity was identical to reported values for larger peptides that encompassed the peptide used here (31). This is the first report identifying a single amino acid being critical in the binding of gp160 to calmodulin, although several groups have published studies on the effects of mutating the C terminus of gp160. Tenceza et al. (18) reported that changing several basic residues to negatively charged glutamic acid residues and single hydrophobic residues changed to polar residues all reduced calmodulin binding and the lytic function of these peptides. Other reports have shown that gp160 binds to calmodulin and that C-terminal truncations of gp160 do not (40), but these mutations are more likely to involve changes in tertiary structure and thus are probably less specific than the single point mutation.

Although the mechanism by which gp160 up-regulates calmodulin is not known, the simplest explanation is that gp160 may be acting as a calmodulin sink, binding available calmodulin, and that cells compensate by increasing expression of calmodulin. Calmodulin expression correlates with increased expression of wild type gp160 but is unchanged with expression of gp160A835W, clearly suggesting that calmodulin is a critical signaling molecule in gp160-enhanced apoptosis.

Expression of gp160 has been reported to induce apoptosis without stimulation by FAS antibody (40). Induction of apoptosis was dependent on calmodulin binding to gp160, as cells expressing C-terminal truncations of as few as five amino acids did not bind calmodulin and did not show increased apoptosis. This supports the basic findings of our earlier studies (19) except that we did not observe differences in basal apoptosis between gp160 and mock-transfected cells. There are, however, significant differences between the two studies (ranging from cell lines used to methods of transfection and measurements of apoptosis) that make direct comparisons impossible.

There are also reports of HIV-1 and simian immunodeficiency virus proteins other than gp160 that increase apoptosis, including Vpu (41), Nef (42), and Tat (43). Whereas the proposed mechanisms that these proteins use to enhance apoptosis vary, they include FAS ligand up-regulation (Nef), caspase 8 up-regulation (Tat), and all converge on FAS-mediated apoptosis. These reports, coupled with the data presented in this report, leave open the possibility that calmodulin plays a central role in mediating the effects of each of these proteins.

Currently, there are no known calmodulin-dependent enzymes directly involved in the FAS pathway, but data presented here suggest that at a minimum, calmodulin binding is required to mediate gp160-enhanced apoptosis. It remains to be determined whether calmodulin-dependent enzyme activity is also essential in this process. Calmodulin-dependent processes have been implicated in glutamate- (44), glucocorticoid- (45), tumor necrosis factor-, and UV light (46)-dependent apoptosis. Furthermore, there are important potential sites for Ca2+/calmodulin action downstream of caspase 3 activation, such as death-associated protein kinase (47–49). Expression of gp160 has also been reported to increase [Ca2+]i, (50) which would promote calmodulin binding to target proteins and potentially increase the activity of the Ca2+/Mg2+-dependentendonuclease responsible for DNA cleavage in apoptosis (10). We hypothesize that gp160 binds to calmodulin and increases calmodulin expression, resulting in recruitment of specific proteins to a membrane complex that enhances the apoptotic potential of the cell. Interestingly, there is considerable sequence homology between the death domains of many apoptosis-inducing proteins and gp41 (19), providing an additional potential mechanism for the recruitment of apoptotic mediating proteins to the membrane.

In conclusion, these investigations provide strong evidence that gp160 expression enhances the cellular response to anti-FAS and increases caspase 3 activity and that these events require calmodulin binding to gp160. The possibility that it is the increase in calmodulin expression that is mediating the effects of gp160 on apoptosis opens a new avenue in the study of HIV interaction with an infected cell. The use of calmodulin antagonists and/or caspase inhibitors may provide useful tools in future studies of viral replication and infectivity, ultimately leading to improved therapies for AIDS and/or effective vaccine development.

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