Casp8p41 generated by HIV protease kills CD4 T cells through direct Bak activation

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Previous studies have shown that human immunodeficiency virus (HIV) protease cleaves procaspase 8 to a fragment, termed Casp8p41, that lacks caspase activity but nonetheless contributes to T cell apoptosis. Herein, we show that Casp8p41 contains a domain that interacts with the BH3-binding groove of pro-apoptotic Bak to cause Bak oligomerization, Bak-mediated membrane permeabilization, and cell death. Levels of active Bak are higher in HIV-infected T cells that express Casp8p41. Conversely, targeted mutations in the Bak-interacting domain diminish Bak binding and Casp8p41-mediated cell death. Similar mutations in procaspase 8 impair the ability of HIV to kill infected T cells. These observations support a novel paradigm in which HIV converts a normal cellular constituent into a direct activator that functions like a BH3-only protein.

Introduction

Apoptosis reflects the interplay between three groups of proteins: caspases, inhibitor of apoptosis (IAP) proteins, and Bcl-2 family members (Taylor et al., 2008; Strasser et al., 2011). Caspases play two previously recognized roles in this process (Earnshaw et al., 1999; Taylor et al., 2008), transducing signals such as death receptor ligation or mitochondrial cytochrome c release into protease activity (initiator caspases) and cleaving a wide variety of cellular constituents to yield the apoptotic phenotype (effector caspases). Some of these cysteine proteases are inhibited by X chromosome–linked IAP (Taylor et al., 2008; Fulda and Vucic, 2012). Moreover, Bcl-2 family members regulate the mitochondrial cytochrome c release that initiates caspase 9 activation. In particular, the proapoptotic Bcl-2 family members Bak and Bak, which are responsible for mitochondrial outer membrane (MOM) permeabilization (MOMP), are inhibited by binding to antiapoptotic family members such as Bcl-2 and Mcl-1, and are activated by BH3-only proteins through either direct interactions (Kim et al., 2009; Gavathiotis et al., 2010; Dai et al., 2011; Czabotar et al., 2013) or neutralization of antiapoptotic family members (Llambi et al., 2011; Strasser et al., 2011).

Viruses have evolved several strategies for impacting cellular apoptotic pathways. These include the expression of IAP proteins such as Op-IAP and antiapoptotic Bcl-2–like proteins such as Epstein-Barr virus BHLF1 (Miller, 1999; Galluzzi et al., 2008). Indeed, study of these proteins has informed current understanding of viral pathogenicity as well as apoptotic pathway regulation. HIV, however, has not previously been shown to impact the core apoptotic machinery directly.

HIV causes death of infected CD4 T cells in three different ways: by triggering pyroptosis during abortive infection (Doitsh et al., 2010), activating an integrase-initiated DNA damage response in cells that integrate the virus (Cooper et al., 2013), or inducing apoptosis in cells that are productively infected, i.e., that produce progeny virions. During HIV production, HIV protease is active in the cytoplasm and at the cell membrane (Kaplan et al., 1994), where it cleaves both viral and host substrates (Ventoso et al., 2001). Moreover, expression of HIV protease is known to induce apoptosis (Baum et al., 1990; Blanco et al., 2003), although the process also

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requires procaspase 8 (Nie et al., 2002; Nie et al., 2008). Curiously, procaspase 8 is directly cleaved by HIV-1 protease between F355 and F356, generating a 41-kD N-terminal fragment, termed Casp8p41, that lacks the catalytic cysteine of an active caspase (Nie et al., 2002; Nie et al., 2008). Nonetheless, protease inhibitor–resistant HIV isolates that replicate but do not cause CD4 T cell decline in patients (discordant responses) contain HIV protease variants that are impaired in their ability to generate Casp8p41 (Natesampillai et al., 2010), which indicates the importance of Casp8p41 in T cell demise during productive HIV infection. Further studies have shown that Casp8p41 traffics to mitochondria (Algeciras-Schimnich et al., 2007; Sainski et al., 2011) and requires procaspase 9 to induce apoptosis (Sainski et al., 2011). How Casp8p41, as a catalytically inactive caspase fragment, activates caspase 9 to induce apoptosis has remained unclear.

In the present study, we show that Casp8p41 binds the Bak BH3-binding groove, leading to Bak oligomerization, Bak-mediated MOMP, and cell death. These observations not only provide new insight into the mechanism of T cell death during productive HIV infection, but also suggest a new paradigm in which a protein that ordinarily does not bind Bel-2 family members can be converted into a direct activator to affect apoptosis.

Results and discussion

Casp8p41-induced death requires Bak

To gain mechanistic insight into Casp8p41-mediated killing, we screened for lentiviral shRNAs that diminish Casp8p41-induced cell death (Fig. S1 A). Because EGFP-Casp8p41 induces death within 24 h (Sainski et al., 2011), cells expressing both EGFP-Casp8p41 and RFP from the shRNA constructs at 48 h must have been altered in a way that renders Casp8p41 less toxic. Of 27,000 shRNAs tested, 1,558 were at least threefold higher in RFP/EGFP double-positive cells compared with background. Of these overrepresented shRNAs, only three (FADD, procaspase 9, and Bak) target proapoptotic components of the core apoptotic machinery. Because procaspase 9, which is involved in HIV-induced T cell death (Sainski et al., 2011), is downstream of mitochondria (Earnshaw et al., 1999; Taylor et al., 2008), we focused on FADD and Bak. Casp8p41 induced similar levels of apoptosis in FADD-deficient and parental Jurkat cells (unpublished data). In contrast, siRNA-mediated Bak knockdown diminished Casp8p41 killing, which indicates an important role for Bak in Casp8p41-induced death (Fig. S1, B and C).

Casp8p41 binds Bak

Based on the observation that Casp8p41, like a BH3-only protein, translocates to mitochondria while inducing apoptosis (Algeciras-Schimnich et al., 2007; Sainski et al., 2011), we assessed the possibility that Casp8p41 and Bak might directly interact. For these studies we initially immobilized recombinant His-tagged Bak lacking the transmembrane domain (His6 BakΔTM) on a surface plasmon resonance (SPR) chip and assessed binding of GST-Casp8p41. Binding occurred in a dose-dependent manner, with an equilibrium dissociation constant (Kd) of 16 ± 5 nM (Fig. 1, A and C; and Fig. S2 A), which is tighter than BakΔTM binding to Bim under comparable conditions (Dai et al., 2011). In contrast, full-length procaspase 8 fused to GST-bound BakΔTM 100-fold less tightly (Fig. 1, B and C; and Fig. S2 B), potentially explaining why full-length procaspase 8 does not independently induce apoptosis.

Casp8p41 contains a latent Bak activator domain

Using protein threading (Wu and Zhang, 2008) and multiple molecular dynamic simulations (57 independent 10-ns simulations with a time step of 1 fs) performed according to published protocols (Dai et al., 2011; Pang et al., 2012), we generated a three-dimensional model of Casp8p41 in order to gain insight into residues of Casp8p41 involved in the interaction with Bak. This model suggested that amino acids 141–160 of Casp8p41 adopt a loop conformation in their free state and an α-helical conformation when bound to the Bak BH3-binding groove (Fig. 2, A and B). Even though this Casp8p41 region exhibits only 35% sequence similarity to classical BH3 domains, the spatial positions of residues critical for Bak binding resemble those of a BH3 domain (Fig. 2 A, bottom). In contrast, in full-length procaspase 8, amino acids 141–160 are buried beneath the C-terminal domain, which folds back on the rest of the protein (Yu et al., 2009). Thus, the ability of HIV-1 protease to proteolytically remove the procaspase 8 C terminus, exposing a loop we term the “latent Bak activator domain” in Casp8p41, accounts for the preferential binding of Casp8p41 but not procaspase 8 to Bak.
Figure 2. The latent Bak activator domain in Casp8p41 interacts with the BH3-binding groove in Bak. (A) Sequence alignment of Bim BH3, Puma BH3, Noxa BH3, and Casp8p41. Multiple molecular dynamics simulations revealed that the three BH3 domains form an α-helix in the Bak BH3-binding groove. Key interactions of the three BH3 domains with the Bak groove involving two hydrophobic residues are highlighted in green (e.g., L141Puma and L148Puma) and one anionic residue in red (e.g., D146Puma). Residues of the latent Bak activator domain spatially approximate these critical BH3 domain residues. (B) Close-up view of the multiple molecular dynamics simulation-refined model of the activator domain of Casp8p41 (green) binding the Bak BH3-binding groove. (C) Immobilized GST-Casp8p41 was exposed to 125 nM His6-Bak WT or His6-BakΔTM R127E. Representative of n = 3. (D) SPR analysis of GST fused to Casp8p41, Casp8p41 V150A/L157A, or Casp8p41 V150E/L157K (400 nM) binding to immobilized His6-Bak ΔTM. Representative of n = 3. (E) Summarized results from three independent experiments (**, P < 0.01). (F) Peptides used in G and H. (G) Immobilized His6-Bak ΔTM was exposed to 400 nM Casp8p41 latent Bak activator peptide, Bim BH3 peptide, or Casp8p41 N-terminal peptide. Representative of n = 3. (H) Summarized results from three independent experiments (***, P < 0.001). (I) Pulldowns from 293T cells transfected with empty vector, HA-Casp8p41, HA-Casp8p41 V150A/L157A, or HA-Casp8p41 V150E/L157K along with S peptide–Bak (left) or S peptide–empty vector (right). Representative of n = 4. Error bars indicate SD.
Figure 3. Casp8p41 facilitates Bak oligomerization and Bak-mediated MOMP. [A] Dextran-FITC–loaded liposomes composed of MOM lipids were incubated with 50 nM His6-BakΔTM in the absence or presence of 200 nM GST, GST-Casp8p41, or GST-Casp8p41 V150E/L157K at 37°C for 30 min. Bim BH3 peptide served as a positive control. After bismaleimidohexane cross-linking, samples were subjected to SDS-PAGE and blotted for Bak. [B] After
Further simulations predicted that Val^{150} and Leu^{157} in the latent Bak activator domain of Casp8p41 (Fig. 2, A and B) dock in two previously described hydrophobic holes in the BH3 binding groove (Dai et al., 2011; Pang et al., 2012). To test this model, we mutated residues in Bak or Casp8p41 predicted to be critical for the interaction. When Bak Arg^{127}, a conserved residue in the BH3-binding grooves of all Bcl-2 family members (Dai et al., 2011), was mutated to Glu, Casp8p41 binding diminished markedly (Fig. 2 C), which implicates the Bak BH3-binding groove in Casp8p41 binding. Likewise, Casp8p41 V150E/L157K bound Bak much less tightly than wild type (wt) binding groove in Casp8p41 binding. Likewise, Casp8p41 (Dai et al., 2011), was mutated to Glu, Casp8p41 binding due to the BH3-binding grooves of all Bcl-2 family members (wt) construct. Simulations to maintain affinity for Bak, bound indistinguishably from the wt construct (Fig. S2 C), highlighting the importance of the latent Bak activator domain in this binding. In contrast, a more conservative Casp8p41 V150A/L157A mutant, which was predicted by the simulations to maintain affinity for Bak, bound indistinguishably from the wt construct (Fig. 2 C). Size exclusion chromatography demonstrated that Casp8p41 also induced higher-order Bak oligomers (Fig. 3 B).

When release of FITC-labeled dextran from liposomes composed of MOM lipids was assessed (Kuwana et al., 2005; Dai et al., 2011), Casp8p41 had no effect on these liposomes by itself but nonetheless increased Bak-mediated release (Fig. 3, C and D). In contrast, Casp8p41 V150E/L157K, which exhibits diminished Bak binding (Fig. 2, D and E) and oligomerization (Fig. 3 A), induced no additional FITC-dextran release above that seen with GST alone (Fig. 3 E). Likewise, Casp8p41 increased the ability of Bak to release cytochrome c from mitochondria, whereas Casp8p41 V150E/L157K did not (Fig. 3, G and I). Importantly, this MOMP depended on the presence of Bak in mitochondria and was not observed in mitochondria from Bak$^{-/-}$ or Bax$^{-/-}$ cells (Fig. 3, H and J).

In additional experiments, we tracked Bak activation using a well-described conformation-sensitive antibody. Like Jurkat cells treated with agonistic anti-Fas antibody (Fig. 4 A), cells transfected with EGFP-Casp8p41 exhibited increased binding of this antibody (Fig. 4 B). Casp8p41-positive peripheral blood mononuclear cells from viremic HIV-infected patients also displayed increased binding of the activation-sensitive anti-Bak antibody relative to Casp8p41-negative cells from the same patients (Fig. 4, C and D).

Collectively, these results indicate that Casp8p41 induces Bak oligomerization and enhances Bak-mediated MOMP, leading to cytochrome c release in vitro, whereas Casp8p41 V150E/L157K is impaired in these functions. Moreover, the presence of Casp8p41 tracks with the activated Bak conformation in vitro and in vivo.

**Mutations in the latent Casp8p41 Bak activator domain impair killing by HIV**

If these observations are pertinent to Casp8p41-mediated killing, the V150E/L157K mutation should diminish apoptosis, whereas the V150A/L157A mutation with preserved Bak binding should not. To test these predictions, we initially transfected plasmids encoding wt EGFP-Casp8p41, the V150A/L157A mutant, or the V150E/L157K mutant into Jurkat T cells and assessed death by annexin V binding 6 h later. Wt Casp8p41 killed 36% of the cells in which it was expressed, and Casp8p41 V150E/L157K killed fewer cells (17%, P < 0.05 vs. wt), whereas Casp8p41 V150A/L157A induced killing similar to wt (28%, P > 0.05 vs. wt; Fig. 5, A and C). Similar results were observed at different time points (Fig. S3 A) and when death was assessed by TUNEL (Fig. 5, B and D). Despite these differences in killing, all Casp8p41 variants trafficked to mitochondria normally (Fig. S3, B and C).
In further experiments, procaspase 8-deficient Jurkat I9.2 cells were transfected with empty vector or plasmids encoding EGFP fused to various procaspase 8 constructs and subsequently infected with vesicular stomatitis virus (VSV) pseudotyped HIV-1 reporter virus (HXB, Δvpu, Δvpr). Viral replication in the various transfected (EGFP+) cells was similar (Fig. S3, E–G). When viability of the transfected (EGFP+) cells was assessed on day 3 after HIV infection, procaspase 8-deficient cells sustained negligible death (82% viable), whereas cells expressing EGFP fused to wt procaspase 8 or the C360S (catalytically inactive) mutant were killed equally (35% and 38% viable, respectively; Fig. 5 E), which confirms that procaspase 8 is required for HIV-induced killing but that caspase 8 catalytic activity is not (Algeciras-Schimnich et al., 2007; Nie et al., 2008).

Consistent with the protein binding data, HIV-induced killing was diminished in cells expressing EGFP–procaspase 8 V150E/L157K compared with the wt construct (61% vs. 35% viable on day 3, P = 0.005; Fig. 5 F). Not surprisingly, HIV-induced killing was not completely eliminated by the V150E/L157K mutation, as other HIV-encoded proteins known to induce cell death such as Tat and nef are still expressed, and infected cells killing by activation of the DNA damage response or accumulation of reverse transcripts can still occur (Doitsh et al., 2010; Cooper et al., 2013). Nonetheless, the decrease in cell killing when HIV-producing cells express EGFP–procaspase 8 V150E/L157K at levels similar to wt EGFP–procaspase 8 (Fig. S3 D) demonstrates that HIV-induced death depends substantially upon the ability to generate Casp8p41 that can bind the Bak BH3-binding groove. Consistent with this conclusion, EGFP–procaspase 8 V150A/L157A, which generates Casp8p41 V150A/L157A that binds Bak almost as strongly as wt Casp8p41 (Fig. 2), supports death of infected cells at a rate that approaches that of cells expressing wt procaspase 8 (Fig. 5 F, 48% vs. 35% viable, P = 0.09).

Collectively, these observations suggest a novel and unexpected mechanism by which Casp8p41, a catalytically inactive caspase fragment, activates the mitochondrial apoptotic pathway. In particular, our results show for the first time that Casp8p41 interacts with Bak to induce Bak oligomerization, Bak-induced MOMP, and Bak-mediated T cell killing (Figs. 1, 3, and 5). Further experiments have traced the Casp8p41–Bak interaction to a domain in Casp8p41 that is unmasked when the C-terminal end of procaspase 8 is removed by HIV protease (Fig. 2). Importantly, a synthetic peptide containing this activator domain binds to Bak (Fig. 2 G) to trigger subsequent events. Further, charge-altering mutation of two critical residues in the activator domain markedly diminishes binding of Casp8p41 to Bak ex vivo (Fig. 2, D and E) as well as T cell killing by Casp8p41 (Fig. 5, A–D) or HIV (Fig. 5 F), which confirms the importance of the Casp8p41-induced Bak activation mechanism described here.

These results have important implications for understanding HIV-induced T cell death. Although HIV-1 can undoubtedly kill both infected and bystander CD4 T cells via multiple mechanisms, our observation that killing of productively infected cells is diminished by critical mutations in the Casp8p41 activator domain (Fig. 5 F) suggests that Casp8p41 plays a prominent role in the death of cells productively infected with HIV. These results also provide an explanation for the clinical observation that impaired Casp8p41 production is associated with smaller declines in CD4+ cell counts (Natesampillai et al., 2010).

The present observation that a domain of the Bcl-2 family member generated or unmasked during cell signaling can serve as a direct activator to trigger mitochondrial apoptosis also provides a new paradigm in apoptotic regulation. Previous studies of Bax and Bak activation have focused exclusively on the role of BH3-only proteins (Cheng et al., 2001; Letal et al., 2002; Walensky et al., 2006; Dai et al., 2011; Llambi et al., 2011; Czabotar et al., 2013; Moldoveanu et al., 2013). Our observation that proapoptotic Bak can be directly activated by Casp8p41 raises a question of whether similar unmasking of latent activator domains in other proteins is used by additional viruses or other cell death stimuli as well.
Materials and methods

Plasmids and peptides

Casp8p41 and procaspase 8 were cloned into pEGFP-C1, pcDNA3-HA, or pGEX (Nie et al., 2008). BakΔTM (aa 1–186) was cloned into pGEX-4T-1 or pSPN (Dai et al., 2011). Mutations introduced using site-directed mutagenesis (Agilent Technologies) were confirmed by sequencing. The Casp8p41 activator peptide (DMNLDFIEEMKVRILGKLDLKRVCAG), Casp8p41 N-terminal control peptide (MDFSRLYDIQEQLDSEDLASLKh), and Bim BH3 peptide (RPEIWIAGEIKDGFYNYYARKKF; Dai et al., 2011) were synthesized in the Mayo Clinic Proteomics Research Center.

Figure 5. Mutations in the latent Bak activator domain impair killing by Casp8p41 or HIV. (A and B) Flow cytometry data from Jurkat T cells transfected with empty vector or EGFP fused to Casp8p41, Casp8p41 V150A/L157A, Casp8p41 V150E/L157K, or procaspase 8 [Casp8] and stained 6 h later with Annexin V [A] or TUNEL [B]. Numbers on the top left indicate percentages of EGFP+ cells that are Annexin or TUNEL positive. Representative of n = 3. (C and D) Pooled data from three independent experiments measuring Annexin V [C] or TUNEL [D] positivity in EGFP+ cells. Error bars indicate ±1 SD. (E) I9.2 cells transfected with EGFP, EGFP–procaspase 8, or EGFP–procaspase 8 C360S were infected with VSV-G–HIV-1, and the EGFP-positive cells were monitored for viability over time. (F) I9.2 cells reconstituted with EGFP fused to procaspase 8, procaspase 8 V150E/L157K, or procaspase 8 V150A/L157A were infected with VSV-G–HIV-1, and the EGFP-positive cells were monitored for cell viability over time. *, P < 0.05; **, P < 0.01 by linear regression. Error bars indicate SD.
Cell culture
Jurkat cells, the Jurkat variant I9.2 lacking procaspase 8, and 293T cells were obtained from American Type Culture Collection and cultured as instructed by the supplier. Fibrolasts from wt, Bak−/−, Bak−/+, and Bak+/−/Bak−/− mouse embryos were obtained from Z. Dong (Mayo Clinic, Augusta, GA). The Bak−/− mice were generated by deletion of exons 3–6 of the murine Bak gene (Wei et al., 2001). In both cases, neomycin cassettes were inserted by homologous recombination.

Transfections and infections
Jurkat and I9.2 cells were transfected with 2 µg of DNA per 10^5 cells using a lipofectamine method. Jurkat cells, the Jurkat variant I9.2 lacking procaspase 8, and 293T cells (10^6) were infected with a library containing 27,000 shRNAs in fresh medium.

Gustafsson, GA). The Bak protein was detected by immunoblotting, without stripping between blots. Primary antibodies used were: anti-HA peroxidase high-affinity 3F10 (rat; Roche) or anti–S- Peptide HRP (mouse; EMD Millipore).

Protein expression and purification
Plasmids for GST- and His6-tagged proteins were transformed into Escherichia coli BL21 or DH5α by heat shock, grown to an optical density of 0.8, and induced with 1 mM IPTG for 24 h at 16°C or 3°C at 37°C. Bacteria were harvested and thawed on ice, suspended in calcium- and magnesium- free Dulbecco’s PBS containing 0.1% Triton X-100, 2 µg/ml apronin, 10 µg/ml leupetin, 2 µg/ml pepstatin, and 1 mM PMSF (GST-tagged proteins) or 150 mM NaCl containing 10 mM Tris-HCl, pH 7.4, [TS buffer] and 1 mM PMSF (His-tagged proteins); and sonicated three times for 15 s/min on ice. His6-tagged proteins were purified using Ni2+-NTA-agarose (EMD Millipore). Alternatively, GST-tagged proteins were purified with glutathione-agarose (Thermo Fisher Scientific).

SPR
All proteins for SPR were further purified by fast protein liquid chromatography (FPLC) on Superdex 2000, concentrated in a centrifugal concentrator (Centricon; EMD Millipore), dialyzed against Biacore buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.05 mM EDTA, and 0.005% [wt/vol] Polysorbate 20), and stored at 4°C for <48 h before use. Binding assays were performed at 25°C on a Biacore 3000 biosensor using GST-Casp8p41 or His6-BakΔTM immobilized on a CM5 chip (GE Healthcare). Ligands were injected at 30 µl/min for 1 min in Biacore buffer. Bound protein was allowed to dissociate in Biacore buffer for 50 µl/min for 10 min and then desorbed with 2 M MgCl2. Binding kinetics were derived using BIAX evaluation software (Biacore; GE Healthcare).

Preparation of FITC-Dextran lipid vesicles
1-Palmitoyl-2-oleooyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleooyl-sn-glycero-3-phosphoethanolamine, i,-phosphatidylglycerol, cholesterol, cardiolipin, and 18:1 DGS-NTA(Ni) at a weight ratio of 36:22:9:8:20:5 were dried as thin films in glass test tubes under nitrogen and then under vacuum for 16 h. 50 mg of FITC-labeled dextran 10 (F-d10; Molecular Probes) was encapsulated in lipid in 1 ml of 20 mM Hepes, 150 KCl, pH 7.0, by sonication for 15 min times 10 min in 100-nm polycarbonate membrane. Gel filtration on Sephacryl S-300 HR (GE Healthcare) was used to remove untrapped F-d10. Phosphate was determined by colorimetric assay (Abcam).

Liposome release assay
Release of F-d10 from large unilamellar vesicles (LUVs) was monitored by fluorescence quenching using a fluorimetric plate reader. Purified His6- Bak with or without other proteins was added to LUVs (final lipid concentration, 10 µg/ml) in 96 well plates, which were then incubated at 37°C and monitored (excitation, 485 nm; emission, 538 nm) every 10 s. The equation [Fsample − Fblank]/[FTriton − Fblank]) × 100] was used to calculate F-d10 release, where Fsample, Fblank, and FTriton are fluorescence of reagent-, buffer-, and Triton X-100-treated LUVs.

Analytical gel filtration
Purified His6-Bak and Casp8p41 proteins were mixed in CHAPS buffer (1% CHAPS, 1% glycerol, 150 mM NaCl, 5 mM DTT, and 20 mM Hepes, pH 7.5) at 23°C for 1 h. 200-µl samples were subjected to FPLC at 4°C on a Superdex 200 (GE Healthcare). Fractions (500 µl) were analyzed by SDS-PAGE followed by immunoblotting. Molecular markers (Sigma-Aldrich) in CHAPS buffer were run through the same column.

Cytochrome c release
Purified GST-Casp8p41, or GST-Casp8p41 EK were dialyzed against mitochondria buffer (150 mM KCl, 5 mM MgCl2, 1 mM EGTA, and 25 mM Hepes, pH 7.5). Mitochondria purified from wt, Bak−/−, Bak−/+, or Bak−/−/Bak−/− of mice were incubated with the indicated proteins at 23°C for 1 h. After centrifugation (10,000 g for 15 min), supernatants and pellets were analyzed by immunoblotting.

Clinical HIV samples
Blood was obtained from five HIV-infected, viroemic patients according to a Mayo Clinic Institutional Review Board approved protocol. After patients signed informed consent, peripheral blood mononuclear cells were isolated by Ficoll gradient, fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% Nonidet P-40 and 5% bovine serum albumin, and stained with a monoclonal antibody specific for Casp8p41 directly conjugated with Mix-n-Stain CF 640R (Sigma-Aldrich) overnight at 4°C.
Cells were costained with the primary and secondary antibodies using Alex Fluor 488 and Alexa Fluor 594 conjugates. The fluorescence was imaged using a Zeiss confocal microscope (Axio Observer Z1). Image J was used to measure the intensity of both the primary and secondary antibody signal in randomly selected cells. Image J was used to measure the intensity of both the primary and secondary antibody signal in randomly selected cells.

### Computational model of Bak in complex with the Casp8p41 activator domain

The starting structure of the complex was generated by manually docking the activator domain (residues 142–162) into the α-helical conformation into the vacated B3-H3-binding groove of Bak (residues 21–183) as described by Dai et al., 2011; Pang et al., 2012). This manual docking placed V150D-methionine in the proximity of L114K and L118Q, and L157 casp8p41 close to V129K and L184K. All His, Glu, Asp, and Lys residues were treated as HIP, GLU, ASP, ARG, and LYS, respectively. The topology and coordinate files of the docking-generated Casp8p41–Bak complex were generated by the PREP, LINK, EDIT, and PARM modules of the AMBER 5.0 program (Pearlman et al., 1999). The complex was refined by energy minimization using the SANDER module of the AMBER 5.0 program with a dielectric constant of 1.0 and 500 cycles of steepest-descent minimization followed by 10,000 cycles of conjugate-gradient minimization. The energy-minimized complex was solvated with 6,947 TIP3P water molecules (Jorgensen et al., 1983), leading to a system of 23,767 atoms. The water molecules were obtained from solvating the complex using a preequilibrated box of 216,000 TIP3P molecules, whose hydrogen atom charge was set to 0.4170, where any water molecule was removed if it had an oxygen atom closer than 2.2 Å to any solute atom or a hydrogen atom closer than 2.0 Å to any solute atom, or if it was located further than 10.0 Å along the x, y, or z axis from any solute atom. The solvated complex system was energy-minimized for 100 cycles of steepest-descent minimization followed by 100 cycles of conjugate-gradient minimization to remove close van der Waals contacts in the system, then heated from 0 to 300 K at a rate of 10 K/ps under constant temperature and volume, and finally simulated independently with a unique seed number for initial velocities at 300 K under constant temperature and pressure using the PMEMD module of the AMBER 8.0 program (Case et al., 2005) with a dielectric constant of 1.383. The PMEMD module of the AMBER 8.0 program was used to simulate the complex.

### Statistical analysis

A one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests was used for single point time samples in experiments containing more than two variables. For experiments in which all aliquots were taken from the same sample over multiple days, linear regression models were used. P values for linear regression used the F test to compare the elevations of the best-fit lines using the software GraphPad Prism. All but one of the experiments have been repeated at least three times independently.

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Casp8p41 generated by HIV protease kills CD4 T cells through direct Bak activation

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Due to an error during the production process, an incorrect version of Fig. 3 was published. Specifically, incorrect images were included for the top two panels of part I, and several figure parts contained minor formatting and labeling errors. A corrected version of the figure is shown below.

Figure 3. Casp8p41 facilitates Bak oligomerization and Bak-mediated MOMP. (A) Dextran-FITC–loaded liposomes composed of MOM lipids were incubated with 50 nM His₆-BakΔTM in the absence or presence of 200 nM GST, GST-Casp8p41, or GST-Casp8p41 V150E/L157K at 37°C for 30 min. Bim BH3 peptide served as a positive control. After bismaleimidoehexane cross-linking, samples were subjected to SDS-PAGE and blotted for Bak. (B) After His₆-BakΔTM was incubated with GST or GST-Casp8p41 in buffer containing 1% (wt/vol) CHAPS, fractions from size exclusion chromatography were blotted for Bak and GST. Representative of n = 3. (C) FITC-dextran release over time from FITC-dextran–loaded liposomes treated as in A. Representative of n = 3. (D) Summarized results from three independent experiments at 300 s of FITC-dextran release from liposomes. (E) FITC-dextran release from liposomes treated with 50 nM His₆-BakΔTM in the presence of 200 nM GST-Casp8p41, GST-Casp8p41 V150E/L157K (EK), or GST. 100% release in D and E was determined by treating liposomes with Triton X-100. Error bars indicate ±1 SD from three independent experiments (*, P < 0.05; ***, P < 0.001). (F–J) After mitochondria (F) from wt (G), Bax⁻/⁻/Bak⁻/⁻ double knockout (H), Bax⁻/⁻ (I), or Bak⁻/⁻ (J) MEFs were incubated with 200 (1), 500 (2), or 1,000 nM (3) of purified protein for 1 h, sedimented, and washed, the supernatants and pellets were blotted for cytochrome c (Cyto C) and, as a control, the mitochondrial matrix protein Hsp60. Bim BH3 (200 nM), which induces CytoC release in the presence of either Bak or Bax, served as a positive control. Representative of n = 3.

The html and pdf versions of this article have been corrected. The error remains only in the print version.