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## Bax cleavage is mediated by calpain during drug-induced apoptosis

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The anti-apoptotic molecule Bcl-2 is located in the mitochondrial and endoplasmic reticulum membranes as well as the nuclear envelope. Although its location has not been as rigorously defined, the pro-apoptotic molecule Bax appears to be mainly a cytosolic protein which translocates to the mitochondria upon induction of apoptosis. Here we identify a protease activity in mitochondria-enriched membrane fractions from HL-60 cells capable of cleaving Bax which is absent from the cytosolic fraction. Bax protease activity is blocked *in vitro* by cysteine protease inhibitors including E-64 which distinguishes it from all known caspases and granzyme B, both of which are involved in apoptosis. Protease activity is also blocked by inhibitors against the calcium-activated neutral cysteine endopeptidase calpain. Partial purification of the Bax protease activity from HL-60 cell membrane fractions by column chromatography revealed that a calpain-like activity was the protease responsible for Bax cleavage. In addition, purified calpain enzymes cleaved Bax in a calcium-dependent manner. Pretreatment of HL-60 cells with the specific calpain inhibitor calpeptin effectively blocked both drug-induced Bax cleavage and calpain activation, but not PARP cleavage or cell death. These results suggest that calpains and caspases are activated during drug-induced apoptosis and that calpains, along with caspases, may be involved in modulating cell death by acting selectively on cellular substrates.

**Keywords:** drug-induced apoptosis; calpain; Bax cleavage; HL-60 cells; mitochondrial membranes

### Introduction

Apoptosis is regulated by the activity of several intracellular cysteine proteases of the ICE family (caspases) (Cohen, 1997; Porter *et al.*, 1997). All known caspase family members are located in the cytosol as zymogens where they become activated by apoptotic stimuli to form a signal transduction cascade (Cohen, 1997; Salvesen and Dixit, 1997). The substrates for some caspases are known and include pro-interleukin-1 $\beta$  and interferon- $\gamma$  inducing factor for caspase-1, poly(ADP-ribose) polymerase (PARP), D4-GDI and U1-70 kDa for caspase-3 and lamin A for caspase-6 (Friedlander *et al.*, 1996; Gu *et al.*, 1996;

Nicholson *et al.*, 1995; Na *et al.*, 1996; Casciola-Rosen *et al.*, 1996; Orth *et al.*, 1996). It is widely believed that caspases are responsible for the destruction of cellular architecture leading to the distinct morphological appearance of apoptotic cells (Porter *et al.*, 1997).

Calpains are another family of cysteine proteases of which two isozymes are ubiquitously expressed,  $\mu$ - and m-calpain. These proteases also reside primarily in the cytosol, but translocate to cellular membranes where they appear to become activated (Kawasaki and Kawashima, 1996). Indeed, many calpain substrates are located at or near cellular membranes and include the cytoskeleton associated proteins  $\alpha$ - and  $\beta$ -spectrin and the  $\alpha$ ,  $\beta$  and  $\gamma$  isoforms of the membrane associated enzyme protein kinase C (Croall *et al.*, 1986; Kishimoto *et al.*, 1989). Other substrates not localized at membranes include the nuclear protein cyclin D1 (Choi *et al.*, 1997). Calpains have been implicated in many cellular processes including platelet aggregation, cytoskeletal reorganization during endocytosis and exocytosis and in several models of cell death including some instances of necrosis and apoptosis (Kawasaki and Kawashima, 1996; Geeraerts *et al.*, 1991; Lee *et al.*, 1991; Nichols *et al.*, 1994; Nath *et al.*, 1996; Squier and Cohen, 1997).

Recent data have illustrated the central role of mitochondria in initiating cell death. Upon induction of apoptosis this organelle undergoes a series of changes that are crucial to the death program. One event, the mitochondrial permeability transition (PT), results from the opening of a large pore that is formed by a group of proteins located in the inner and outer mitochondrial membranes at sites where these membranes come into contact with each other (reviewed by Reed, 1997). The PT leads to a disruption in the mitochondrial membrane potential ( $\Delta\psi$ ) which in several apoptotic systems appears to be a very early, obligatory step in the death program (reviewed by Zamzami *et al.*, 1997) and release of a protease ('apoptosis inducing factor', AIF) into the cytosol from the mitochondrial intermembrane space which is capable of initiating nuclear apoptotic events *in vitro* (Susin *et al.*, 1996). Another molecule released from the intermembrane space into the cytosol and capable of producing apoptotic changes is cytochrome c (Kluck *et al.*, 1997; Yang *et al.*, 1997). Cytochrome c has been shown *in vitro*, along with caspase-9 and the human homolog of CED-4 in the presence of ATP, to be critical for activating the zymogen form of caspase-3 in the cytosol initiating the execution phase of the apoptotic cascade (Li *et al.*, 1997).

The anti-apoptotic molecule Bcl-2 is located in the membranes of the mitochondria, endoplasmic reticulum and nuclear envelope (Hockenbery *et al.*, 1990; Krajewski *et al.*, 1993; Akao *et al.*, 1994). The pro-

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apoptotic molecule Bax, however, appears to be localized primarily in the cytosol but upon induction of apoptosis rapidly translocates to the mitochondria (Hsu *et al.*, 1997; Wolter *et al.*, 1997; unpublished observations). Members of the Bcl-2 family play key roles in the apoptotic events occurring at the mitochondria. Bcl-2 and Bcl-X<sub>L</sub> can block the disruption of the mitochondrial membrane potential as well as release of AIF and cytochrome c into the cytosol (Susin *et al.*, 1996; Decaudin *et al.*, 1997; Kim *et al.*, 1997; Kluck *et al.*, 1997; Yang *et al.*, 1997; Zamzami *et al.*, 1997). Conversely, overexpression of Bax results in translocation of cytochrome c from the mitochondria to the cytosol in both yeast and mammalian cells (Manon *et al.*, 1997; Rosse *et al.*, 1998). In addition Bax, Bcl-2 and Bcl-X<sub>L</sub> can form channels in synthetic lipid membranes (Antonsson *et al.*, 1997; Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997). However, the ability of Bax to form channels can be inhibited by Bcl-2 (Antonsson *et al.*, 1997). The fact that Bcl-2 is located at the mitochondria and that Bax translocates to this organelle upon induction of apoptosis, may help explain why mitochondria are in a critical position to control the fate of the cell.

Previously, we reported the appearance of an 18 kDa protein during drug-induced apoptosis in B-cells from chronic lymphocytic leukemia (B-CLL) patients which was recognized by anti-Bax antibody (Thomas *et al.*, 1996). Up-regulation of the 18 kDa Bax protein (p18) occurs following drug-induced apoptosis in normal human B-cells, in several hematopoietic cell lines including HL-60 promyelocytic leukemia cells, in several non-hematopoietic cell lines and in the presence of cycloheximide (data not shown). The fact that protein synthesis was not required for the appearance of the 18 kDa Bax protein and that it increased in amount over time in apoptotic cell cultures suggested proteolysis of the 21 kDa form of Bax (p21).

We report here that Bax is cleaved by a calcium-activated cysteine protease, calpain, in HL-60 cells during drug-induced apoptosis. Using an *in vitro* cleavage assay with HL-60 cell fractions enriched with mitochondrial membranes, we demonstrate that cleavage is blocked by inhibitors of cysteine proteases and calpains. In addition, we show that purified calpain enzymes cleave Bax in a calcium dependent manner. Finally, pretreatment of HL-60 cells with the specific calpain inhibitor calpeptin effectively blocked both drug-induced Bax cleavage and calpain activation, yet PARP cleavage and cell death still occurred.

## Results

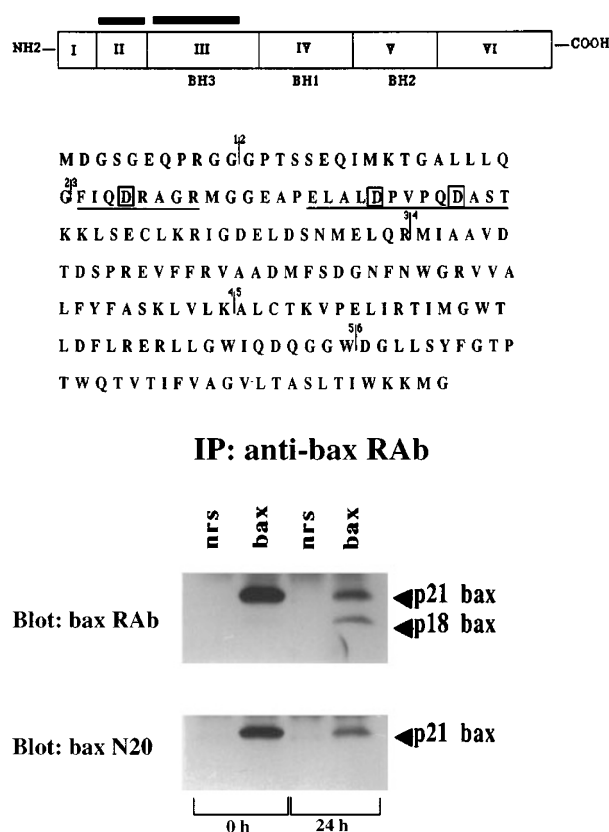
### *p21 Bax is cleaved at the N-terminus during apoptosis*

To determine whether p21 Bax was cleaved to form the 18 kDa protein, we induced apoptosis in HL-60 cells with the topoisomerase I inhibitor 9-AC and immunoprecipitated Bax from total cell lysates from drug-treated and untreated cells using anti-Bax specific antibody (Krajewski *et al.*, 1994) (Figure 1). The immunoprecipitates were analysed on SDS-PAGE and immunoblotted with antibodies raised to epitopes

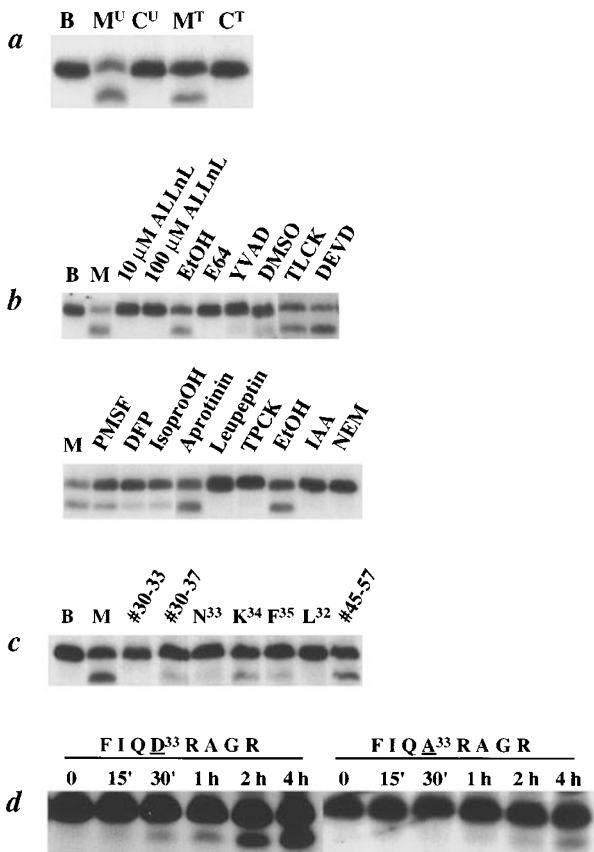
encoded by exon 2 (N20) or exon 3 (RAB) (Krajewski *et al.*, 1994) of the 21 kDa Bax protein (Figure 1). The 21 kDa Bax protein present at time zero and 24 h after drug treatment was detected with both antibodies recognizing either determinants (Figure 1). However, the 18 kDa protein present in cell lysates at 24 h after treatment could only be detected by the antibody specific for determinants encoded by exon 3. This result indicated cleavage in the N-terminus of Bax removing epitopes encoded by exon 2, but retaining the epitopes encoded by exon 3.

### *Bax cleavage enzyme activity in HL-60 membrane extracts*

To identify the protease responsible for p21 Bax cleavage, we developed an *in vitro* assay to detect Bax cleavage enzyme activity (Figure 2). <sup>35</sup>S-methionine-labeled Bax was generated as a substrate by coupled *in vitro* transcription and translation of a full-length human Bax cDNA and then incubated with different cell extracts. Since Bcl-2 is localized to the membranes of mitochondria (Hockenbery *et al.*, 1990;



**Figure 1** Characterization of p21 Bax cleavage. (a) Schematic diagram of the 6 coding exons (I–VI) of the Bax cDNA shows the location of the highly conserved Bcl-2 homology regions 1, 2 and 3 (BH1–3). Solid bars denote the epitopes encoded by exon 2 or exon 3 recognized by anti-Bax antibodies N20 (Santa Cruz) or RAB (Krajewski *et al.*, 1994), respectively. The underlined amino acids within exon 3 of the human Bax  $\alpha$  sequence at positions #30–37 and #45–57 represent the 8- and 13-mer peptide sequences used to inhibit Bax protease activity. The Asp at positions Asp<sup>33</sup>, Asp<sup>48</sup> and Asp<sup>53</sup> are boxed. (b) Bax was immunoprecipitated from HL-60 cells at time zero (before treatment) or 24 h after 9-AC treatment using normal rabbit serum (NRS) or anti-Bax RAB and immunoblotted with RAB or the N20 antibody. Films were exposed for 1 min



**Figure 2** Characterization of the Bax cleavage enzyme. (a) The location of Bax cleavage activity was examined by incubating <sup>35</sup>S-methionine-labeled Bax with buffer alone (B) or heavy membrane (M) or cytosol (C) extracts from untreated HL-60 cells at time zero (U) or 12 h after 9-AC treatment (T) as described in the Materials and methods. (b) The protease inhibitor profile of Bax cleavage was characterized using heavy membrane extracts that were preincubated with buffer alone (B) or with one of the following protease inhibitors: aprotinin (2 μg/ml), ALLnL (10 and 100 μM), DFP (100 μM), E-64 (10 μM), IAA (5 mM), NEM (5 mM), PMSF (100 μM), TLCK (100 μM), TPCK (100 μM), 1 μM of the tetrapeptide specific for caspase-1 (Ac-YVAD-cmk) or 10 μM of the tetrapeptide aldehyde specific for caspase-3 (Ac-DEVD-CHO) as described in the Materials and methods. Controls consisted of membrane extracts preincubated with the same volume of the inhibitor vehicles DMSO, EtOH and IsoproOH as well as buffer alone with <sup>35</sup>S-methionine-labeled Bax (B). (c) To identify the cleavage site, heavy membrane extracts (10 μg) were incubated alone (M) or with 4 mM each of peptides spanning residues #45–57 (ELALDPVPQDAST) or various 8-mer peptides spanning residues #30–37 containing no substitutions (FIQDRAGR), or substitutions at Asp<sup>33</sup> to Asn<sup>33</sup> (N<sup>33</sup>), at Arg<sup>34</sup> to Lys<sup>34</sup> (K<sup>34</sup>), Ala<sup>35</sup> to Phe<sup>35</sup> (F<sup>35</sup>) and at Gln<sup>32</sup> to Leu<sup>32</sup> (L<sup>32</sup>) or the tetrapeptide spanning residues #30–33 (FIQD). Buffer alone with <sup>35</sup>S-methionine-labeled Bax (B) served as the negative control. (d) Time course of cleavage of <sup>35</sup>S-methionine-labeled Bax substrate containing the wild-type sequence (FIQD<sup>33</sup>RAGR) or the mutant sequence (FIQA<sup>33</sup>RAGR) incubated with heavy membrane extracts for various periods of time as indicated. For (a–d) the products of each reaction were examined by SDS–PAGE and autoradiography as described in the Materials and methods. Abbreviations used: DFP, diisopropyl fluorophosphate; E-64, trans-Epoxy succinyl-L-leucylamido-(4-guanidine)butane; IAA, iodoacetic acid; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; TLCK, N- $\alpha$ -p-Tosyl-L-lysine chloromethyl ketone; TPCK, N-Tosyl-L-phenylalanine chloromethyl ketone; Ac-YVAD-cmk, Ac-Tyr-Val-Ala-Asp-chloromethyl ketone; Ac-DEVD-CHO, Ac-Asp-Glu-Val-Asp-CHO (aldehyde); DMSO, dimethylsulfoxide; EtOH, ethanol and IsoproOH, isopropanol

Krajewski *et al.*, 1993; Akao *et al.*, 1994) and Bax translocates to his organelle (Hsu *et al.*, 1997; Wolter *et al.*, 1997; unpublished observations), we reasoned that the Bax protease activity might reside in the same compartment. HL-60 cells at time zero or 12 h after 9-AC treatment (T) were fractionated into cytosol (C) or heavy membranes (M) enriched for mitochondria and incubated with labeled Bax substrate (Figure 2a). Cytosol extracts from both untreated and drug-treated HL-60 cells lacked protease activity. Heavy membrane extracts from drug-treated cells (M<sup>T</sup>) contained protease activity that produced cleavage of p21 Bax to the 18 kDa protein (Figure 2a). Surprisingly, enzyme activity was also present in heavy membrane extracts prepared from control untreated (M<sup>U</sup>) HL-60 cells. Since the 18 kDa Bax cleavage product has never been detected *in vivo* in the absence of drug treatment (Figure 1) (Thomas *et al.*, 1996; unpublished data), this result suggested that detection of enzyme activity in membranes from untreated HL-60 cells *in vitro* could be due to protease activation or the loss of an inhibitor of protease activity during the cell fractionation procedure.

#### Protease inhibitor profile

To characterize the enzyme activity present in HL-60 heavy membrane extracts, protease inhibitors were screened for their ability to block p21 Bax cleavage (Figure 2b). Enzyme activity was completely inhibited by the cysteine class of protease inhibitors E-64, leupeptin, IAA, NEM and substantially blocked by the tetrapeptide inhibitor of caspase-1, Ac-YVAD-cmk (Lazebnik *et al.*, 1994). The activity was not inhibited by the specific tetrapeptide inhibitor of caspase-3, Ac-DEVD-CHO (Nicholson *et al.*, 1995). Reports have shown that caspases are insensitive to E-64, but sensitive to NEM or IAA (Black *et al.*, 1989; Thornberry *et al.*, 1992; Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Martins *et al.*, 1997). The calpain inhibitor I, ALLnL, at 10 and 100 μM also completely prevented Bax cleavage. In contrast, the serine class of protease inhibitors aprotinin, DFP, PMSF and TLCK did not block activity. However, the serine protease inhibitor TPCK did block activity. TPCK can block the activity of some types of endoproteases that cleave after phenylalanine residues and can inhibit calpain-like activity *in vitro* (Pinter *et al.*, 1992). As discussed below, phenylalanine appears to be part of the cleavage site within Bax. The results of these inhibitor studies suggest that Bax cleavage is mediated by a membrane-associated, calpain-like cysteine protease which differs from the known caspases present in the cytosol and nuclei of HL-60 cells undergoing apoptosis such as caspase-2, -3 and -6 (Martins *et al.*, 1997; Polverino and Patterson, 1997).

#### Cleavage site of Bax

We next investigated the cleavage site within Bax. The most likely cleavage site is located within exon 3 (Figure 1) which would be consistent with the loss of 3 kDa in apparent molecular weight from 21 to 18 kDa. Since proteases in apoptotic pathways such as the caspases and the serine proteases granzyme B

cleave at Asp-X bonds (Cohen, 1997; Poe *et al.*, 1991), we examined whether cleavage might occur at one of three Asp residues located at positions Asp<sup>33</sup>, Asp<sup>48</sup> or Asp<sup>53</sup> using peptides spanning the putative cleavage sites from amino acids #30–37 or amino acids #45–57, respectively (Figure 2c). The peptide #30–37 significantly inhibited Bax cleavage whereas the peptide #45–57 did not suggesting that the cleavage site was within the region spanning residues #30–37 of Bax. Next we examined if substitutions of residues at positions 32–35 would have an effect on Bax cleavage activity. Surprisingly, mutant 8-mer peptides containing Asn<sup>33</sup> substituted for Asp<sup>33</sup> or with Leu<sup>32</sup> substituted for Gln<sup>32</sup> completely inhibited protease activity. Bax cleavage was also markedly reduced compared to the heavy membrane extract control, but not completely inhibited with peptides containing Lys<sup>34</sup> for Arg<sup>34</sup> or Phe<sup>35</sup> for Ala<sup>35</sup> substitutions. Finally, when the tetrapeptide sequence FIQD, spanning residues #30–33, was tested for inhibition it completely blocked Bax cleavage activity in heavy membrane extracts. These data show that substitution of amino acids at positions 32–35 within 8-mers spanning the 30–37 region of Bax resulted in peptides that were still able to block Bax cleavage, albeit with varying degrees of efficacy, indicating a less essential role for these residues in recognition of Bax by the protease. In contrast, the tetrapeptide FIQD, which is complementary to the Bax sequence at residues 30–33, completely inhibited cleavage suggesting that this region may play an important role in recognition of Bax by the protease.

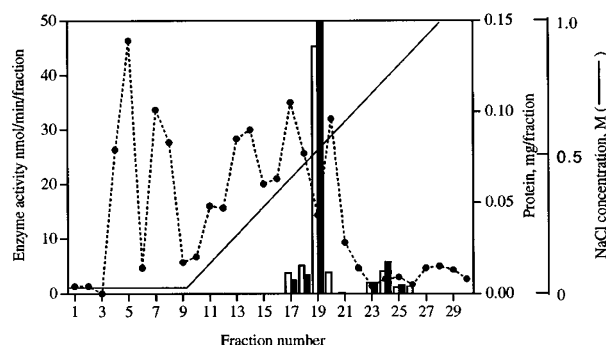
#### Mutation of Asp<sup>33</sup> to Ala inhibited Bax cleavage

Since caspases require an Asp in the P1 position for effective cleavage of their substrates (Cohen, 1997), we wanted to further investigate the requirement for Asp<sup>33</sup> in the sequence FIQD as a site for cleavage of Bax by the protease. We generated Bax with an Asp<sup>33</sup> to Ala<sup>33</sup> substitution by site-directed mutagenesis. As expected, when wild-type <sup>35</sup>S-labeled Bax containing Asp<sup>33</sup> was used as a substrate *in vitro* Bax cleavage was efficient and detectable by 15 min with the amount of cleaved substrate increasing significantly over the 4 h interval (Figure 2d). When mutant [<sup>35</sup>S]Bax containing Ala<sup>33</sup> was used as a substrate, Bax cleavage was inefficient such that the 18 kDa cleavage product was detectable only after 1 h of incubation. These results together with the fact that the mutant Asn<sup>33</sup> (N<sup>33</sup>) peptide completely blocked cleavage (Figure 2c), indicate that the Asp<sup>33</sup> residue is not absolutely required for the protease activity to cleave Bax *in vitro*; this differs from the strict requirement of caspases for Asp in the P1 position (Cohen, 1997).

#### Partial purification of the Bax cleavage enzyme from HL-60 membrane extracts

To identify the protease responsible for Bax cleavage, heavy membrane extracts prepared from HL-60 cells were subjected to a Mono Q anion-exchange chromatography. The fractions were monitored for enzyme activity using a fluorogenic peptide, Ac-FIQD-7-amino-4-methyl-coumarin (AMC), that contains the putative Bax cleavage site. As shown in Figure 3, a single peak of enzyme activity was detected that eluted

at approximately 500 mM NaCl. The fraction containing the highest enzyme activity was found to correlate with a protein band on a silver stained gel around 80 kDa which was absent from samples lacking protease activity (data not shown). This size is similar to the large subunit of both  $\mu$ - and m-calpain (Kawasaki and Kawashima, 1996). Based on the results of the protease inhibitor profile which demonstrated that the Bax cleavage activity was completely inhibited by cysteine protease inhibitors and that the size corresponded to the large subunit of  $\mu$ - and m-calpain, we suspected that a calpain might be the enzyme responsible for Bax proteolysis. To test this, the fractions from the column were reassayed for calpain enzyme activity using the fluorogenic calpain substrate N-Suc-Leu-Tyr-AMC (Kenney *et al.*, 1994). As illustrated in Figure 3, a single peak of enzyme activity was also detected in the same fraction corresponding to the activity observed with Ac-FIQD-AMC. This activity was characterized with calpain selective inhibitors as summarized in Table 1. The activity as measured by cleavage of Ac-FIQD-AMC was inhibited to a similar extent by calpain inhibitor I (ALLnL) and calpain inhibitor II (ALLM). Likewise, cleavage of N-Suc-Leu-Tyr-AMC was also blocked to a similar extent by both ALLnL and ALLM. These results taken together are consistent



**Figure 3** Purification of Bax cleavage activity from HL-60 heavy membrane fractions. Isolation of heavy membranes, extraction of proteins and ion-exchange chromatography using a Mono Q anion exchange column to partially purify the activity was carried out as described in the Materials and methods. The Bax cleavage activity using both Ac-FIQD-AMC (open bar) and N-Suc-Leu-Tyr-AMC (shaded bar) as substrates was determined for each fraction. The enzyme activity is expressed as nanomoles of AMC released/min/fraction. Protein content (dotted line) is expressed as mg/fraction of each fraction as determined by using the BCA reagent (Pierce). The NaCl gradient (0.02–1.0 M) is represented by the solid line

**Table 1** Inhibition of partially purified Bax cleavage enzyme against fluorogenic substrates in the presence of calpain inhibitors

Substrate	Inhibitor	Calpain-like protease activity (% of control)
Ac-FIQD-AMC	ALLnL	20.7 ± 2.2
	ALLM	22.8 ± 2.7
N-Suc-Leu-Tyr-AMC	ALLnL	11.1 ± 0.8
	ALLM	11.9 ± 0.7

The percent calpain-like protease activity was calculated as the ratio of fluorescence units with inhibitor (each at 100  $\mu$ M) over the fluorescence units without inhibitor (control). The data represent the average of three experiments and are expressed as means  $\pm$  s.e.m.

with the protease which cleaves Bax being a calpain-like enzyme.

*Identification of Bax as substrate for calpain in vitro*

To determine whether Bax was a substrate for calpain, purified  $\mu$ - and m-calpain enzymes were tested in the *in vitro* Bax cleavage assay for their ability to cleave <sup>35</sup>S-labeled p21 Bax to p18. As shown in Figure 4a, both  $\mu$ - and m-calpain were able to efficiently cleave Bax in a Ca<sup>2+</sup>-dependent manner. Fifty percent cleavage of p21 Bax to p18 occurred with both  $\mu$ - and m-calpain at 100  $\mu$ M Ca<sup>2+</sup> and 500  $\mu$ M Ca<sup>2+</sup>, respectively.

Specific calpain inhibitors were tested for their ability to block Bax cleavage activity in heavy membrane extracts. The activity was substantially inhibited in the presence of 1, 10 and 100  $\mu$ M of the calpain inhibitors ALLnL and ALLM (Figure 4b). Both of these calpain inhibitors are also capable of inhibiting the proteasome (Rock *et al.*, 1994). To rule out the possibility of proteasome-mediated cleavage of Bax, we used the proteasome-specific inhibitor lactacystin (Fenteany *et al.*, 1995). Lactacystin failed to significantly block Bax proteolysis at 1, 10 and 100  $\mu$ M (Figure 4b). These findings further support the notion that Bax cleavage is mediated by a calpain.

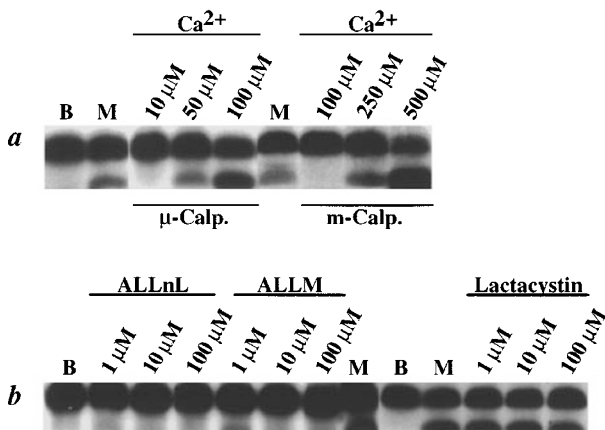
*Calpain mediated Bax cleavage in HL-60 cells*

As noted above in Figure 2a, membranes prepared from non-apoptotic control HL-60 cells contained Bax cleavage activity which converted p21 Bax to p18. To determine whether calpain mediated Bax cleavage under these circumstances, cytosol (C) and heavy membranes (M) were prepared from HL-60 cells in the absence or presence of the inhibitors leupeptin and EGTA, both of which block calpains, in the lysis buffer. Immunoblots of the cell lysates were probed with an anti-Bax antibody and an antibody which recognizes the 30 kDa subunit common to  $\mu$ - and m-

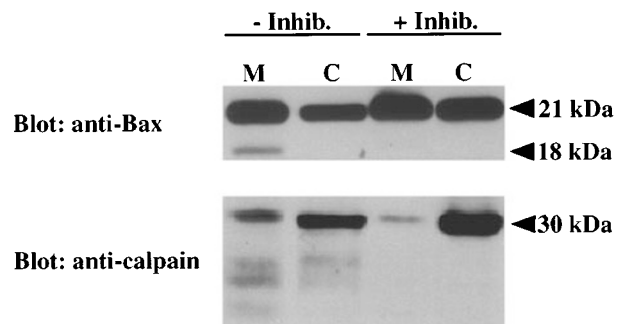
calpain. As demonstrated in Figure 5, cell fractionation in the absence of calpain inhibitors resulted in cleavage of p21 Bax to p18 at the heavy membranes whereas no cleavage was observed when cell fractionation was performed in the presence of inhibitors. Similarly, the 30 kDa small subunit of calpain underwent proteolysis to several different break-down products (BDPs) under conditions where cell fractionation was done in the absence of inhibitors. Autolysis of calpain subunits is used as a general marker for calpain activation (Kawasaki and Kawashima, 1996; Nath *et al.*, 1996; Molinari and Carafoli, 1997). These BDPs were not present when fractionation was carried out with leupeptin and EGTA. These observations taken together indicate that a calpain-like enzyme mediates Bax cleavage *in vivo* in HL-60 cells and explains why extracts from non-apoptotic cells possessed Bax cleavage activity in Figure 2a.

*Calpain inhibitors block 9-AC induced Bax and calpain cleavage, but not PARP cleavage or cell death in HL-60 cells*

To further confirm that Bax cleavage was mediated by a calpain *in vivo*, the cell permeable calpain-specific inhibitor calpeptin (benzyloxycarbonyldipeptidyl; Z-Leu-nLeuH, Mehdi, 1991) was tested for its ability to block both 9-AC-induced Bax cleavage in HL-60 cells and proteolysis of calpain to the BDPs indicative of activation of this enzyme (Kawasaki and Kawashima, 1996; Nath *et al.*, 1996; Molinari and Carafoli, 1997). Figure 6 is a representative experiment in which HL-60 cells untreated (data not shown) or pretreated with 30  $\mu$ M calpeptin alone showed little to no p18 Bax cleavage product at 24 h (Figure 6). In contrast, cultures challenged with the drug 9-AC alone for 24 h showed significant conversion of p21 Bax to p18 (44.8  $\pm$  2.0%) as well as extensive proteolysis (46.9  $\pm$  8.5%) of the 30 kDa calpain subunit to the BDPs. However, HL-60 cells pretreated with calpeptin prior to 9-AC drug challenge showed a reduced amount of conversion of p21 Bax to p18 (11.1  $\pm$  2.9%) and proteolysis of the 30 kDa calpain



**Figure 4** Characterization of Bax cleavage by calpains. (a) The ability of  $\mu$ - or m-calpain to cleave <sup>35</sup>S-methionine-labeled Bax was examined using various concentrations of CaCl<sub>2</sub> and compared to the endogenous activity in heavy membrane extracts (M) or buffer alone (B) (b) The ability of calpain inhibitors (ALLnL and ALLM) or a proteasome inhibitor (lactacystin) to block Bax cleavage by heavy membrane extracts were examined as described in the Materials and methods. Buffer alone (B) served as the negative control. Reactions were analysed as described in Figure 2



**Figure 5** Calpain-mediated Bax cleavage in HL-60 cells. To determine if Bax cleavage was the result of calpain activation during subcellular fractionation, non-apoptotic HL-60 cells were harvested and subjected to the fractionation procedure as described in the Materials and methods. Heavy membrane (M) and cytosol (C) fractions were generated either in the absence (-Inhib.) or the presence (+ Inhib.) of 10  $\mu$ g/ml leupeptin and 1 mM EGTA in the lysis buffer. The blots were probed with a polyclonal anti-Bax antibody or a monoclonal anti-calpain antibody recognizing the conserved 30 kDa small subunit of  $\mu$ - and m-calpain

subunit to BDPs ( $11.3 \pm 1.5\%$ ) after 24 h. Similar results were obtained in HL-60 cells that were pretreated with the calpain inhibitors ALLM or leupeptin (data not shown).

The ability of calpeptin pretreatment of HL-60 cells to block death was examined by measuring trypan blue exclusion at 24 h following 9-AC treatment. Treatment with 9-AC alone resulted in 41% ( $40.6 \pm 4.3\%$ ) of the cells scoring positive for uptake of the dye compared to 42% ( $41.5 \pm 3.9\%$ ) for cells that had been pretreated with calpeptin prior to drug treatment. Similar results were obtained when HL-60 cells were pretreated with calpain inhibitors ALLM and leupeptin (data not shown). Failure to block 9-AC induced cell death in HL-60 cells pretreated with calpain inhibitors prompted us to assess caspase activity by assaying for PARP cleavage. PARP is a substrate for caspase-3 and similar members of the caspase family and has been used as a general marker for caspase activity in several models of apoptosis (Cohen, 1997). As shown in Figure 6, pretreatment of HL-60 cells with calpeptin alone did not activate caspase activity since the amount of the full-length 116 kDa protein was similar at 0 and 24 h. However, when the cells were induced to undergo apoptosis with 9-AC treatment for 24 h, none of the full-length PARP remained compared to 0 h. Pretreatment of cells with calpeptin followed by 9-AC treatment for 24 h also resulted in the complete loss of full-length PARP compared to 0 h, similar to what was observed when cultures were treated with 9-AC alone. This result indicated that caspase-3-like proteases are activated during 9-AC induced death and that calpeptin does not prevent caspase activation. It appears that 9-AC induced apoptosis of HL-60 cells activates both calpains and caspases which are each capable of acting on specific cellular substrates.

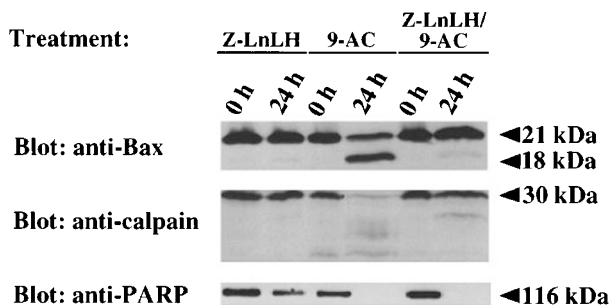
## Discussion

We have shown that Bax is a substrate for cleavage during drug-induced apoptosis of human B-leukemia cells *in vivo*. A consequence of this cleavage is the formation of p18 Bax homodimers (Thomas *et al.*,

1996). Using an *in vitro* assay and column chromatography, we have identified the Bax cleavage enzyme activity in mitochondria-enriched membrane extracts from HL-60 cells as a calpain, a calcium-activated neutral cysteine endopeptidase. The protease inhibitor profile of calpains is consistent with the profile of the Bax cleaving activity in that both are cysteine proteases due to their mutual inhibition by E-64, leupeptin, IAA, and NEM. Both activities are also inhibited by calpain inhibitors ALLnL and ALLM, but not by the specific proteasome inhibitor lactacystin (Fenteany *et al.*, 1995). The partially purified enzyme activity from extracts of HL-60 heavy membranes was active on the synthetic calpain substrate N-Suc-Leu-Tyr-AMC and was blocked by specific calpain inhibitors. Also, the molecular weight of a major protein band on a silver-stained gel in the fraction showing the highest Bax cleavage activity using synthetic peptide substrate was consistent with the size of the large subunit of  $\mu$ - and m-calpains (data not shown). Both  $\mu$ - and m-calpain share a common 30 kDa small subunit, but have different 80 kDa large subunits (Kawashima and Kawashima, 1996). Furthermore, Bax could be cleaved *in vitro* from 21 kDa to 18 kDa with purified  $\mu$ - and m-calpain. Finally, preparation of heavy membrane fractions (enriched with mitochondrial membranes) in the presence of calpain inhibitors prevented the appearance of calpain activation fragments as well as the Bax p18 cleavage product. This suggested that the Bax cleavage observed in these extracts was due to calpain activation within cells during subcellular fractionation.

Several lines of evidence suggest that the protease responsible for cleaving Bax is not a caspase. The first observation is the association of the cleavage activity with cell fractions enriched with mitochondria. At present, all known caspases in HL-60 cells are found primarily in the cytosol and nucleus (Martins *et al.*, 1997; Polverino and Patterson, 1997). Second, purified caspase-3 failed to cleave Bax *in vitro* (data not shown) and Ac-DEVD-CHO failed to block Bax cleavage in the *in vitro* assay (Figure 2b) suggesting that caspase-3 is not responsible for Bax cleavage in HL-60 cells. Also, PARP cleavage occurred in apoptotic cells that had been pretreated with the calpain inhibitor calpeptin whereas Bax cleavage did not suggesting that activated caspase-3 was not cleaving Bax *in vivo*. Although Ac-YVAD-cmk could inhibit Bax cleavage *in vitro* at high concentrations, it is unlikely that caspase-1/ICE is responsible for cleavage since purified caspase-1 failed to cleave Bax *in vitro* (data not shown) and does not become activated in HL-60 cells that have been stimulated to undergo apoptosis (Martins *et al.*, 1997; Polverino and Patterson, 1997). These results taken together indicate that Bax is a substrate for a calpain and not a caspase at the mitochondria or other organelles present in heavy membrane fractions. The presence of calpain-like activity at the mitochondria has been documented previously, but relevant substrates *in vitro* or *in vivo* have not been identified to date (Beer *et al.*, 1982; Tavares and Duque-Magalhaes, 1991; Aguilar *et al.*, 1996).

All known caspases cleave at Asp-X sequences and show strong P1 and P4 requirements for cleavage specificity of their substrates (Cohen, 1997; Talanian *et al.*, 1997). In these experiments we have demonstrated



**Figure 6** Bax cleavage, calpain activation and PARP cleavage in the absence or presence of a specific calpain inhibitor in 9-AC treated HL-60 cells. HL-60 cells were pretreated with calpeptin (Z-LnLH) then treated with 9-AC to induce apoptosis as described in the Materials and methods. Whole cell lysates were made at 0 and 24 h after 9-AC treatment and subjected to Western blotting analysis using a polyclonal anti-Bax antibody, a monoclonal antibody that recognizes the conserved 30 kDa small subunit of  $\mu$ - and m-calpain and a monoclonal PARP antibody that recognizes the full-length 116 kDa PARP

that Bax cleavage activity *in vitro* requires Asp<sup>33</sup> because mutation to Ala significantly decreased cleavage activity. Unlike the caspases, however, the substitution of Asp<sup>33</sup> did not completely abrogate substrate cleavage suggesting that this Asp residue is not as critical for enzyme recognition as it is for the caspases to recognize and cleave the appropriate sites within their substrates. The tetrapeptide FIQD modeled on the putative Bax cleavage site completely eliminated cleavage activity *in vitro* indicating that this sequence may be important for recognition of Bax by a calpain. But the fact that peptides containing amino acid substitutions at or surrounding the Asp<sup>33</sup> in the cleavage site decreased with varying efficacy the ability of Bax to be cleaved *in vitro* is consistent with the less stringent sequence requirements of calpains for their substrates relative to the caspases (Wang *et al.*, 1989; Talanian *et al.*, 1997).

At present, the mechanism by which Bax promotes apoptosis is unclear. However, recent data has begun to elucidate some of the potential roles that Bax may play in cell death. It appears that Bax mobilization to the mitochondria is a crucial early step in the death program. Using a fusion protein of Bax and green fluorescent protein (GFP) in mammalian cells, Bax was found to reside diffusely throughout the cytosol but translocated rapidly to the mitochondria when cells were treated with staurosporine (Wolter *et al.*, 1997). Prevention of Bax translocation to the mitochondria in this system by removal of the carboxy terminal tail prevented apoptosis (Wolter *et al.*, 1997).

The structures of the anti-apoptotic protein Bcl-X<sub>L</sub> alone and complexed with the death promoting molecule Bak have been deduced (Muchmore *et al.*, 1996; Sattler *et al.*, 1997) and can be used as a model for Bax. The BH1, BH2 and BH3 regions of Bcl-X<sub>L</sub> (Yin *et al.*, 1994; Boyd *et al.*, 1995; Chittenden *et al.*, 1995; Zha *et al.*, 1996a) were found to form an elongated hydrophobic cleft representing a possible binding site for other Bcl-2 family members (Muchmore *et al.*, 1996). The structure of Bcl-X<sub>L</sub> complexed with Bak demonstrated that an alpha helix containing the BH3 domain of Bak is crucial for binding the hydrophobic groove in Bcl-X<sub>L</sub> (Sattler *et al.*, 1997). In order for binding between these two molecules to take place, this helix would need to undergo a conformational change to allow for the BH3 domain of Bak to bind to the hydrophobic groove in Bcl-X<sub>L</sub> (Muchmore *et al.*, 1996; Sattler *et al.*, 1997). The BH3 region of Bak is similar to that of Bax (Chittenden *et al.*, 1995) suggesting that Bax may undergo a similar structural change for its BH3 region to be involved in heterodimerization with anti-apoptotic molecules (Sattler *et al.*, 1997).

The BH3 domain of Bax is required for heterodimerization with Bcl-2, homodimerization and cell death (Boyd *et al.*, 1995; Chittenden *et al.*, 1995; Zha *et al.*, 1996a,b). Cleavage of Bax results in an 18 kDa form that still retains the BH3 domain (Figure 1). It is possible, however, that cleavage of the 21 kDa form of Bax by a calpain results in structural changes within this molecule, analogous to Bak, which in turn allows for the disruption of interactions with Bcl-2 thereby precluding heterodimerization. We have observed that treatment of HL-60 cells with 9-AC resulted in the inability of Bcl-2 to be co-immunoprecipitated with

Bax suggesting that the molecules were no longer capable of heterodimerizing (data not shown). Having liberated itself from Bcl-2, Bax would then be free perhaps to form p18 homodimers that we have observed associated with 9-AC induced cell death (Thomas *et al.*, 1996). It has been suggested that Bax homodimerization is a crucial event for this molecule to promote apoptosis (Yang and Korsmeyer, 1996; Zha *et al.*, 1996b; Zha and Reed, 1997).

The X-ray and NMR structure of uncomplexed Bcl-X<sub>L</sub> revealed that this protein resembles the membrane insertion domains of certain bacterial toxins especially diphtheria toxin and the colicins (Muchmore *et al.*, 1996). The diphtheria toxin translocation domain is believed to dimerize and form membrane pores (London, 1992; Muchmore *et al.*, 1996). Bcl-X<sub>L</sub>, along with Bcl-2 and Bax, have also shown the capacity to form ion conducting pores in synthetic lipid membranes (Schendel *et al.*, 1997; Minn *et al.*, 1997; Schlesinger *et al.*, 1997; Antonsson *et al.*, 1997). The p18 Bax homodimerization observed during 9-AC induced death may be a prerequisite for pore formation similar to that of diphtheria toxin. It is tempting to speculate that Bax pore formation at the mitochondria may trigger a membrane permeability transition (PT) and consequent disruption of the mitochondrial transmembrane potential ( $\Delta\psi$ ) (Zamzami *et al.*, 1997). Interestingly, the PT is also observed in necrosis where calpain activity at the mitochondria appears to be necessary for PT induction (Aguilar *et al.*, 1996). Alternatively, pores formed by Bax at the mitochondria could result in the release of cytochrome c from the intermembrane space into the cytosol (Kluck *et al.*, 1997; Yang *et al.*, 1997; Jurgensmeier *et al.*, 1998) where it has been shown to be involved in activation of caspases (Devereaux *et al.*, 1997; Li *et al.*, 1997). Supporting this notion is the observation that Bax overexpression in both yeast and mammalian cells results in cytochrome c translocation from the mitochondria to the cytosol (Manon *et al.*, 1997; Jurgensmeier *et al.*, 1998; Rosse *et al.*, 1998).

Calpains have been implicated in several models of apoptosis (Sarin *et al.*, 1993; Squier *et al.*, 1994; Nath *et al.*, 1996; Vanags *et al.*, 1996; Squier and Cohen, 1997). Calpains cleave their substrates in a highly specific, but limited fashion (Wang *et al.*, 1989) resulting in biologically active proteins (Kawasaki and Kawashima, 1996). These proteases appear to be activated at cellular membranes where many of their substrates reside (Kawasaki and Kawashima, 1996). Movement of Bax from the cytosol to the mitochondria (Wolter *et al.*, 1997; Hsu *et al.*, 1997; unpublished observations) would therefore position Bax for limited cleavage at that organelle by an activated calpain.

Induced expression of Bax in Bax-deficient Jurkat cells resulted in an early decline in mitochondrial membrane potential as well as the generation of reactive oxygen species (ROS) (Xiang *et al.*, 1996). These findings suggested that Bax localization at the mitochondria was responsible for initiating a cell death program at that organelle (Xiang *et al.*, 1996). Intriguingly, in this study Z-VAD-fmk inhibited caspase cleavage of nuclear as well as cytosolic substrates and prevented DNA fragmentation but failed to inhibit membrane permeability changes as measured by propidium iodide exclusion. Other



protease inhibitors, including the calpain inhibitors ALLnL and ALLM, also failed to block Bax-induced death in Jurkat cells (Xiang *et al.*, 1996). Pretreatment of HL-60 cells with calpeptin and other calpain inhibitors did not completely abolish calpain activation or Bax cleavage that occurred with 9-AC treatment. Under these conditions 9-AC drug treatment induced PARP cleavage in both the absence and presence of calpeptin suggesting that both calpains and caspases were activated. The presence of both protease activities has also been observed in other apoptotic models (Nath *et al.*, 1996; Vanags *et al.*, 1996). Thus, small amounts of limited proteolysis of Bax by a calpain may be sufficient to set in motion a mitochondrial-based cell death program which directly controls the activation of caspases. Limited cleavage of Bax at the mitochondria by a calpain followed by pore formation may play a key role in the ability of this organelle to serve as the initiator of the downstream execution stage of apoptosis or necrosis. Future studies will examine the molecular function of Bax cleavage at the mitochondria.

## Materials and methods

### Immunoprecipitation and immunoblot analysis

Total cell lysates (200  $\mu$ g) precleared for 2 h with protein Sepharose A (Pharmacia) were immunoprecipitated with 2  $\mu$ g rabbit polyclonal anti-Bax antibody (Krajewski *et al.*, 1994) for 4 h at 4°C, followed by incubation with Sepharose A for 2 h at 4°C. Immunoprecipitates were washed several times with Triton X-100/NP-40 lysis buffer, resuspended in SDS sample buffer, boiled for 5 min and centrifuged briefly at 300 g. Supernatants were electrophoresed on 12% SDS-PAGE gels, transferred to PVDF membranes (Millipore) and immunoblotted as described (Thomas *et al.*, 1996) with human-specific polyclonal rabbit anti-Bax antisera made against peptides corresponding to amino acids #43–61 at 1:1000 (Krajewski *et al.*, 1994) and with a rabbit polyclonal anti-Bax antibody corresponding to amino acids #11–30 used at 1:50 (N20, Santa Cruz). Donkey anti-rabbit IgG (Amersham Life Science) HRP-conjugated secondary antibody was used at 1:2000. Blots were developed using the ECL chemiluminescence detection kit (Amersham Life Science) and membranes were exposed to Hyperfilm-ECL for appropriate time intervals.

### Subcellular fractionation

$2.5-5 \times 10^7$  HL-60 cells were washed twice in ice-cold PBS, resuspended in 2.5–5.0 ml ice-cold lysis buffer (10 mM HEPES pH, 7.4, 5 mM MgCl<sub>2</sub>, 42 mM KCl and 0.32 M sucrose), lysed with a 30 gauge needle until >95% of the cells were trypan blue positive and fractionated by differential centrifugation to yield cytosol (C) and fractions enriched for mitochondrial membranes (heavy membranes, M) as described (Hockenbery *et al.*, 1990). Unless indicated, fractionation was carried out in the absence of protease inhibitors. Where noted, 10  $\mu$ g/ml of leupeptin and 1 mM EGTA were added to the lysis buffer. The heavy membrane fraction was solubilized in 100  $\mu$ l of resuspension buffer (10 mM HEPES, pH 7.4, 5 mM MgCl<sub>2</sub>, 42 mM KCl and 1% Triton X-100). The cytosol was precipitated with 6 volumes of cold acetone. After 20 min at –20°C the cytosol was centrifuged 5 min at 2000 g and the pellet was taken up in 100–250  $\mu$ l of resuspension buffer. Drug treatment consisted of incubating cells for 12 h with 4  $\mu$ M 9-amino-20(s)-camptothecin (9-AC, kindly provided by Dr M Potmesil).

### In vitro cleavage assay

Bax  $\alpha$ , subcloned into pcDNA3, was used for coupled *in vitro* transcription/translation in the presence of [<sup>35</sup>S]methionine (NEN) according to the manufacturer's instructions (TNT, Promega). The Asp<sup>33</sup> of wild-type Bax in pcDNA3 was replaced with Ala<sup>33</sup> using a polymerase chain reaction (PCR) site-directed mutagenesis kit (ExSite, Stratagene) and confirmed by DNA sequence analysis. The <sup>35</sup>S-methionine-labeled Bax substrate (0.5  $\mu$ l) was incubated with 10  $\mu$ g of cytosol or heavy membrane extracts for 2 h at 37°C. For a control, labeled Bax substrate (0.5  $\mu$ l) was incubated with 4.5  $\mu$ l of assay buffer (10 mM HEPES, pH 7.4, 1% Triton X-100). For the cleavage assay using purified calpain enzymes, labeled Bax substrate (0.5  $\mu$ l) was incubated with 100 ng of purified  $\mu$ - or m-calpain (Chemicon) in assay buffer containing different CaCl<sub>2</sub> concentrations in a total volume of 5  $\mu$ l for 1 h at 37°C. Heavy membrane extracts (10  $\mu$ g) incubated with labeled Bax (0.5  $\mu$ l) served as a positive control while assay buffer (4.5  $\mu$ l) incubated with labeled Bax (0.5  $\mu$ l) was used as a negative control. The peptide inhibitor studies used heavy membrane extracts (10  $\mu$ g) incubated with 4 mM of each peptide and labeled Bax (0.5  $\mu$ l) for 2 h at 37°C. For the protease inhibitor profile, heavy membrane extracts (10  $\mu$ g) were preincubated with various concentrations of specific inhibitors or the solvents used for inhibitor solubilization for 45 min at 25°C in assay buffer in a total reaction volume of 4.5  $\mu$ l then 0.5  $\mu$ l of labeled Bax substrate was added and incubated for 2 h at 37°C. All inhibitors were purchased from Sigma with the exception of Ac-YVAD-cmk (Calbiochem), Ac-DEVD-CHO (a gift from Dr A Zychlinsky) and lactacystin (a gift from Dr M Pagano). For the time course analysis of Bax cleavage, a master reaction of 35  $\mu$ l was made consisting of heavy membrane extract, assay buffer and either wild-type or Asp<sup>33</sup> mutant labeled Bax substrate from which 5  $\mu$ l was removed at each time interval and boiled immediately to stop cleavage activity. For all experiments, the total reaction mixtures after incubation were mixed with an equal volume of 2 $\times$ SDS sample buffer, boiled for 5 min at 100°C and analysed on 14% SDS-PAGE gels. Autoradiography was performed for 1–4 h at –70°C.

### Column chromatography and fluorogenic assays

Approximately 4.5 mg of heavy membrane fraction from  $5 \times 10^8$  HL-60 cells was generated as described above. The resulting membrane extract was centrifuged at 16 000 g for 20 min and the pH of the cleared supernatant, which retained virtually all of the activity in the fluorogenic assays (data not shown, see below), was adjusted to pH 7.6 with 1 M Tris-HCl, pH 7.6 to a final concentration of 20 mM Tris-HCl and to a final NaCl concentration of 20 mM. The supernatant was applied to a Mono Q HR 5/5 anion exchange chromatography column (Pharmacia) that had been equilibrated with 20 mM Tris-HCl, pH 7.6, 20 mM NaCl (loading buffer). Following washing with 4 ml loading buffer, a 10 ml linear NaCl gradient from 0.02–1.0 M in the same buffer was used to elute the proteins. Fractions of 500  $\mu$ l were collected at a rate of 1 ml/min. Protein estimated for each fraction was done using the BCA reagent (Pierce). All fractions were assayed for Bax cleavage enzyme activity in a fluorogenic cleavage assay using either the custom Bax peptide Ac-FIQD-7-amino-4-methyl-coumarin (AMC) (Enzyme Systems Products) modeled on the putative Bax cleavage site or the synthetic calpain substrate N-Suc-Leu-Tyr-AMC (Sigma). The fluorogenic assay consisted of 150  $\mu$ M of fluorescent substrate incubated with 10  $\mu$ l of each fraction and assay buffer (10 mM HEPES, pH 7.4, 1% Triton X-100) in a total volume of 100  $\mu$ l for 2 h at 37°C in 96-well plates. For the calpain substrate experiments, 100  $\mu$ M CaCl<sub>2</sub> was

added to the assay buffer. For the inhibitor studies, 100  $\mu$ M of the calpain inhibitors ALLnL and ALLM (both from Chemicon) or the inhibitor vehicle (water) were preincubated with the fractions for 15 min at 25°C followed by the addition of substrate as described (Aguilar *et al.*, 1996). The plate was read in a Perkin Elmer LS50B luminescence spectrometer using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The amount of AMC released was determined from standard curves containing 0.0–1.0 mmol of AMC. To determine the protein content of the fractions with enzyme activity, 5  $\mu$ l of the fractions in the presence of 5%  $\beta$ -mercaptoethanol (Bio-Rad) were resolved on a 12% SDS–PAGE gel and silver stained to visualize protein bands.

#### *Inhibitor studies in HL-60 cells*

HL-60 cells ( $10^6$ /well) were cultured in flat-bottomed 6-well dishes (Nunc) in 3 ml of RPMI-1640 medium (Cellgro) containing 10% fetal calf serum (Atlanta Biologicals), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 1 mM sodium pyruvate and 2 mM glutamine (all from Gibco–BRL) at 37°C for 42 h prior to the addition of calpeptin (Calbiochem). HL-60 cells were either left untreated or pretreated with calpeptin (30  $\mu$ M) or DMSO (calpeptin solvent) for 15 min at 37°C and then were either left untreated, challenged with DMSO or the drug 9-AC (4  $\mu$ M, dissolved in DMSO) for an additional 24 h at 37°C. Cultures were harvested at the time of drug challenge (0 h) or 24 h later and subjected to trypan blue staining in triplicate. The percentage of dead cells was calculated as the number of trypan blue positive cells minus the control values at 24 h under each culture condition. Whole cell protein lysates were prepared for immunoblot analysis as described (Thomas *et al.*, 1996).

#### *Immunoblot analysis*

Quantitation of protein was carried out with the BCA reagent (Pierce). Equal amounts of protein (30  $\mu$ g) in the presence of 5%  $\beta$ -mercaptoethanol (Bio-Rad) were sub-

jected to gel electrophoresis using 8 or 12% SDS–PAGE gels with the gels subsequently transferred to PVDF membranes (Millipore) by electroblotting (Thomas *et al.*, 1996). Immunoblotting was performed as described (Thomas *et al.*, 1996). Human-specific polyclonal rabbit anti-Bax antisera made against peptides corresponding to amino acids #43–61 was used at 1:1000 (Krajewski *et al.*, 1994). A mouse monoclonal anti-calpain antibody (Chemicon) which recognizes the small 30 kDa subunit of  $\mu$ - and m-calpain was used at 1:1000. A mouse monoclonal anti-PARP antibody (clone C2-10, Pharmingen) recognizing the amino terminal 46 kDa DNA binding domain was used at 1:2000. Monoclonal sheep anti-mouse IgG and donkey anti-rabbit IgG (Amersham Life Science) HRP-conjugated secondary antibodies were used at 1:2000. Blots were developed using the ECL chemiluminescence detection kit (Amersham Life Science) and membranes were exposed to Hyperfilm-ECL for appropriate time intervals. Densitometry of immunoblot results was done using a Hewlett Packard Scanjet 2C scanner with analysis by Kodak Digital Science 1D software. The percent cleavage of Bax was calculated as the amount of p18 formed relative to the total amount of intact p21 and p18 after 24 h of drug treatment. The percent calpain proteolysis was measured as the amount of BDPs formed relative to the total amount of intact 30 kDa subunit and BDPs after 24 h of drug treatment. Experiments were done in triplicate and the data are expressed as means  $\pm$  standard error (s.e.m.).

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