N-Terminal Cleavage of Bax by Calpain Generates a Potent Proapoptotic 18-kDa Fragment that Promotes Bcl-2-Independent Cytochrome C Release and Apoptotic Cell Death

Gui Gao and Q. Ping Dou*

Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute, and Department of Biochemistry & Molecular Biology, College of Medicine, University of South Florida, Tampa, Florida 33612

Abstract   Upon apoptosis induction, the proapoptotic protein Bax is translocated from the cytosol to mitochondria, where it promotes release of cytochrome c, a caspase-activating protein. However, the molecular mechanisms by which Bax triggers cytochrome c release are unknown. Here we report that before the initiation of apoptotic execution by etoposide or staurosporin, an active calpain activity cleaves Bax at its N-terminus, generating a potent proapoptotic 18-kDa fragment (Bax/p18). Both the calpain-mediated Bax cleavage activity and the Bax/p18 fragment were found in the mitochondrial membrane-enriched fraction. Cleavage of Bax was followed by release of mitochondrial cytochrome c, activation of caspase-3, cleavage of poly(ADP-ribose) polymerase, and fragmentation of DNA. Unlike the full-length Bax, Bax/p18 did not interact with the antiapoptotic Bcl-2 protein in the mitochondrial fraction of drug-treated cells. Pretreatment with a specific calpain inhibitor calpeptin inhibited etoposide-induced calpain activation, Bax cleavage, cytochrome c release, and caspase-3 activation. In contrast, transfection of a cloned Bax/p18 cDNA into multiple human cancer cell lines targeted Bax/p18 to mitochondria, which was accompanied by release of cytochrome c and induction of caspase-3-mediated apoptosis that was not blocked by overexpression of Bcl-2 protein. Therefore, Bax/p18 has a cytochrome c–releasing activity that promotes cell death independent of Bcl-2. Finally, Bcl-2 overexpression inhibited etoposide-induced calpain activation, Bax cleavage, cytochrome c release, and apoptosis. Our results suggest that the mitochondrial calpain plays an essential role in apoptotic commitment by cleaving Bax and generating the Bax/p18 fragment, which in turn mediates cytochrome c release and initiates the apoptotic execution. J. Cell. Biochem. 80:53–72, 2000.[†]   © 2000 Wiley-Liss, Inc.

Key words: Bax; Bcl-2; calpain; cytochrome c; apoptosis

Apoptosis, a morphologically distinct form of programmed cell death, plays a major role in development, homeostasis, and many diseases including cancer [Song and Steller, 1999]. The process of apoptosis can be divided into three fundamental steps—initiation, commitment, and execution [Reed, 1997]. The cell death pathway can be initiated by many stimuli and insults, including deprivation of growth factors or treatment with radiation, chemotherapeutic agents, or the kinase inhibitor staurosporin [Reed, 1997]. The molecular mechanisms controlling apoptotic commitment are unclear. Results from the most recent experiments have suggested that cellular fate can be determined by Bcl-2 family proteins that are localized in mitochondria [Green and Reed, 1998; Adams and Cory, 1998; Gross et al., 1999]. Apoptotic execution is initiated by activation of effector caspase proteases (such as caspase-3) [Thornberry and Lazebnik, 1998], which in turn cleaves important cellular proteins, including

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poly(ADP-ribose) polymerase (PARP) [Lazebnik et al., 1994], lamin [Lazebnik et al., 1995], DNA-dependent protein kinase [Song et al., 1996], and retinoblastoma protein (RB) [An and Dou, 1996; Tan et al., 1997]. The active caspase-3 also cleaves a caspase-activated deoxyribonuclease inhibitor, resulting in activation of the deoxyribonuclease that is responsible for the internucleosomal fragmentation of DNA [Enari et al., 1998], a hallmark of apoptotic execution [Thornberry and Lazebnik, 1998].

Several members of the Bcl-2 family (such as Bax, Bid, and Bad) promote apoptosis, whereas the other Bcl-2 members (such as Bcl-2 and Bcl-X$_L$) inhibit the cell death process [Green and Reed, 1998; Adams and Cory, 1998; Gross et al., 1999]. The Bcl-2 family proteins also can form homodimers or heterodimers. The ratio of proapoptotic to antiapoptotic proteins in the Bcl-2 family is involved in determination of cellular fate [Green and Reed, 1998; Adams and Cory, 1998; Gross et al., 1999]. In addition to their ratios, the mitochondrial localization of the Bcl-2 family proteins seems essential for their functions. It has been found that the proapoptotic Bcl-2 family members promote, whereas the antiapoptotic members block, the release of cytochrome c from mitochondria to the cytosol [Green and Reed, 1998; Adams and Cory, 1998; Gross et al., 1999]. Once in cytosol, the released cytochrome c, together with Apaf-1, binds and activates caspase-9, which in turn cleaves and activates the effector caspase-3 [Li et al., 1997]. The three-dimensional structures of Bcl-X$_L$ and Bid suggest that these proteins contained domains similar to the pore-forming domains of some types of bacterial toxins [Chou et al., 1999]. Indeed, when added to synthetic membranes, Bcl-2, Bcl-X$_L$, and Bax were able to form ion channels [Schlesinger et al., 1997; Minn et al., 1997]. However, it is unclear whether Bcl-2 family proteins also modulate the pore formation in mitochondria in vivo to mediate cytochrome c release.

It has been shown that in the absence of a death signal, most of the anti- and proapoptotic Bcl-2 members are localized in separate subcellular compartments. Although proapoptotic members mainly remain in cytosol, antiapoptotic members are localized on membranes of mitochondria, endoplasmic reticulum, and nucleus [Gross et al., 1999; Porter, 1999]. After a death signal, the proapoptotic members undergo a posttranslational modification and/or a conformational change, followed by translocation to membranes of cellular compartments, especially mitochondria [Gross et al., 1999; Porter, 1999]. For example, during tumor necrosis factor α- or Fas-induced apoptosis, Bid is first cleaved at its N-terminus by caspase-8, and the carboxy-terminal fragment of Bid is then inserted into the membrane of mitochondria [Li et al., 1998]. In the presence of survival factors, Bad is phosphorylated and sequestered in the cytosol by binding to 14-3-3 proteins. After a death signal, Bad is dephosphorylated and then translocated to mitochondria where it interacts with, and inhibits, Bcl-X$_L$ or Bcl-2 [Zha et al., 1996]. On apoptosis induction, Bax is also translocated to the mitochondria, although the involved molecular mechanisms remain unclear. The Bax translocation process seems to involve its dimerization and conformational change [Gross et al., 1999], which is promoted by some unidentified cytosolic factors [Nmura et al., 1999]. Moreover, removal of the amino-terminal 20 amino acids of Bax enabled it to target mitochondria in vitro in the absence of an activated cytosol [Goping et al., 1998]. Finally, Bid is able to induce the oligomerization and insertion of Bax into the outer mitochondrial membrane during apoptosis [Eskes et al., 2000].

Although cellular mechanisms affecting and regulating apoptosis are still incompletely characterized, protease cascades appear to play a critical role as regulators of apoptotic cell death. Examples of the implicated proteases include caspases [Thornberry and Lazebnik, 1998], proteasome [Dou and Li, 1999; Orlowski, 1999], and calpain [Wang, 2000]. Calpain, the nonlysosomal calcium-activated neutral protease, is present in almost all the cells. There are at least two isoforms of the enzyme, μ- and m-calpain, which differ mainly in the calcium concentration required for their activation in vitro [Carafoli and Molinari, 1998]. Calpain consists of an 80-kDa catalytic subunit and a small 30-kDa subunit that is identical in both isoforms [Carafoli and Molinari, 1998]. In addition, the calpain activity can also be regulated by autoproteolysis and the inhibitor protein calpastatin [Suzuki and Sorimachi, 1998; Kawasaki and Kawashima, 1996; Goll et al., 1992]. Recent studies have shown that calpain activation precedes apoptotic cell death in various cell systems with dif-

ferent apoptosis stimuli, including radiation, dexamethasone, cycloheximide, or *Reovirus* [Waterhouse et al., 1998; Squier and Cohen, 1997; Squier et al., 1999; Debiasi et al., 1999]. Other reports have suggested that calpain is activated by caspase-mediated cleavage of the calpain inhibitor protein calpastatin during the initiation of apoptotic execution [Wang et al., 1998; Porn-Ares et al., 1998]. The activated calpain cleaves several cellular proteins during apoptosis, including the endoplasmic reticulum chaperone glycoprotein GRP94 [Reddy et al., 1999], procaspase-3, and PARP [Mcginnis et al., 1999]. However, whether these cleavage products are required for calpain-mediated cell death has never been shown.

Most recently, one research group reported caspase-dependent activation of calpain that occurred several hours after cleavage of PARP and RB, and fragmentation of DNA [Wood and Newcomb, 1999]. At this late stage of apoptotic execution, the activated calpain cleaved Bax into an 18-kDa fragment (Bax/p18) [Wood and Newcomb, 1999]. These authors, therefore, suggested that excessive or uncontrolled calpain activity play a role downstream of and distinct from caspases in the degradation phase of apoptosis. In contrast to the above report, we report here that calpain-mediated cleavage of Bax into Bax/p18 can also be a very early apoptotic event that occurs in association with release of mitochondrial cytochrome c into the cytosol and occurs several hours before the initiation of apoptotic execution. Both calpain-mediated Bax cleavage activity and Bax/p18 fragment were found in the mitochondrial membrane-enriched fraction of etoposide-treated cells. In addition, the full-length Bax protein, but not the Bax/p18 fragment, was able to interact with the mitochondrial Bcl-2 protein. We hypothesized that Bax/p18 is a potent proapoptotic molecule that mediates cytochrome c release in a Bcl-2-independent fashion. Indeed, pretreatment of cells with a specific calpain inhibitor calpeptin inhibited calpain activation, Bax cleavage, cytochrome c release, and caspase-mediated apoptosis. Furthermore, overexpression of a cloned Bax/p18 cDNA in several human cancer cell lines, including Jurkat T overexpressing Bcl-2 protein, resulted in accumulation of Bax/p18 in mitochondria, which was accompanied by release of cytochrome c and induction of apoptosis. We also found that overexpression of Bcl-2 delayed etoposide-induced calpain activation, Bax cleavage, and cytochrome c–associated apoptotic events.

MATERIALS AND METHODS
Materials

Etoposide (VP-16), staurosporin, proteinase K, RNase, calpain inhibitor I (LLnL), and calpain inhibitor II (LLM) were purchased from Sigma Chemical Co. (St. Louis, MO). Calpeptin, pan-caspase inhibitor (Z-VAD-FMK) and caspase-3 inhibitor III (Ac-DEVD-CMK) were from Calbiochem (La Jolla, CA), and clastolactacystin β-lactone was from BIOMOL (Plymouth Meeting, PA). L-[^{35}S] methionine was purchased from Amersham (Piscataway, NJ).

Cell Culture and Treatment

Jurkat T cells transfected with pcDNA vector alone (Neo) or pcDNA vector containing Bcl-2 cDNA (Bcl-2) were gifts from Dr. Honggong Wang (Moffitt Cancer Center & Research Institute, Tampa, FL). MCF-7, Jurkat T, Neo, and Bcl-2 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. These cells were treated with either 50 μM of VP-16 or 1 μM of staurosporin for the indicated lengths of time in figure legends. For the experiment using a specific calpain inhibitor, Jurkat T cells were pretreated for 1 h with calpeptin at 10 μM, followed by a coincubation with 50 μM of VP-16, as indicated in figure legends.

Subcellular Fractionation

Both cytosolic and mitochondria fractions were isolated at 4°C using a previous protocol [Hockenery et al., 1990] with some modifications. At each time point, cells were washed twice with phosphate-buffered saline (PBS), resuspended in a hypotonic buffer containing 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 5 mM KCl and 1 mM dithiothreitol (DTT), and incubated on ice for 10 min. The cells were then broken by four passes through a 30 G $\frac{1}{2}$ needle fitted on 1-ml syringe, and the lysate was centrifuged at 2,000g for 10 min. The supernatant was collected and centrifuged again at the same condition. The resulting supernatant was then centrifuged at 14,000g for 30 min, followed by collection of both the supernatant and

pellet fractions. The pellet was washed twice with a buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl (pH 7.5), and 1 mM EDTA, and resuspended in a lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl and 0.5% NP-40) as the mitochondria fraction. The supernatant was further centrifuged at 600,000g for 30 min and the resulting supernatant was collected as the cytosol fraction. To prepare a whole cell extract, cells were lysed in the lysis buffer, and the lysate was centrifuged at 14,000g for 30 min. The supernatant was collected as the whole cell lysate.

Bax/p18 cDNA Cloning and Transfection

Bax/p18 cDNA was amplified by PCR with human Bax-α cDNA as template using primer 5′-CGTATAAGCTTATGGATCGAGCAGGGCGA (forward) and 5′-CTATCTCGAGTCAGCCC ATCTTCTTCCAG (reverse) [Wood et al., 1998] and cloned into pcDNA3.0 between Hind III and Xho I restriction enzyme sites. Bax/p18 cDNA was verified by sequencing. MCF-7, Jurkat T, Neo, and Bcl-2 cells were transfected with pcDNA3.0 vector alone or pcDNA3.0 vector containing Bax/p18 or Bax/p21 cDNA using GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's instructions. Briefly, 3 μg of plasmid DNA in 500 μl of RPMI 1640 medium was incubated for 30 min at room temperature with 25 μl of GenePORTER reagent in 500 μl of RPMI 1640 medium. The resultant GenePORTER-plasmid DNA mixture (1 ml) was then added into cells 2 × 10^6 cells in a well of a six-well plate, followed by a 5-h incubation at 37°C in a humidified atmosphere consisting of 5% CO$_2$. After that, 1 ml of RPMI 1640 medium containing 20% fetal calf serum was added, and the cells were further incubated for an additional 43 h before harvest.

Bax Cleavage Assay

[^{35}S]-labeled Bax protein was prepared by using human Bax-α pcDNA3 plasmid and TNT coupled transcription and translation reticulocyte lysate system (Promega, Madison, WI). Bax cleavage assay was performed as described previously [Wood et al., 1998] with some modifications. Briefly, the ^{35}S-labeled Bax protein (1 μl) was incubated with 100 μg of a whole cell extract or a cytosol or mitochondria fraction in an assay buffer (10 mM HEPES, pH 7.4, 5 mM MgCl$_2$, 5 mM CaCl$_2$, and 1 mM DTT), supplemented with ATP-regenerating system (0.1 mg/ml creatine kinase, 100 mM creatine phosphate, and 5 mM ATP) for 2 h at 37°C. For particular experiments, CaCl$_2$ or ATP-regenerating system was not added in the cleavage assay system, as described in the figure legends. For inhibitor studies, 10 μM of calpain inhibitor-1 (LLnL), 10 μM of calpain inhibitor-2 (LLM), 20 μM of pan-caspase inhibitor Z-VAD-FMK, 20 μM of caspase-3 inhibitor Ac-DEVD-CMK, or 20 μM of the specific proteasome inhibitor castolactacystin β-lactone was added in the Bax cleavage system.

Western Blot Analysis

Equal amounts of protein (30−60 μg) from a whole cell lysate, cytosol, or mitochondria fraction were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and then transferred to the nitrocellulose membranes (Schleicher & Schuell, Keene, NH) using a semidry transfer system (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS)-Tween (vol/vol, 0.2%) for 1 h at room temperature and then incubated with the primary antibody overnight at 4°C. After washing three times with PBS-Tween, the membrane was blotted with the secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature and washed again. The protein bands were visualized with the enhanced chemiluminescence system (Amersham). The primary antibodies used were: monoclonal B9 anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500, monoclonal 6A7 anti-Bax antibody (Pharmingen, San Diego, CA) at 1:500, monoclonal anti-Bcl-2 antibody (Dako, Glostrup, Denmark) at 1:500, monoclonal anti-caspase-3 antibody (Oncogene, Cambridge, MA) at 1:200, monoclonal anti-cytochrome C antibody (Pharmingen) at 1:500, polyclonal anti-PARP antibody (Boehringer Mannheim, Indianapolis, IN) at 1:3,000, monoclonal anti-cytochrome oxidase unit ll (COX) antibody (Molecular Probes, Eugene, OR) at 1:200, and monoclonal anti-calpain 30-kDa subunit antibody (Chemicon, Temecula, CA) at 1:500. The secondary antibodies used were anti-mouse IgG-horseradish peroxidase and anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology) at 1:2,000.

Immunoprecipitation-Western Blot Assay

Immunoprecipitation assay was performed as described previously [Fattman et al., 1997]. Briefly, the mitochondria fraction (500 µg of protein) from VP-16-treated or untreated Jurkat T cells was incubated with 5 µg of a monoclonal anti-human Bcl-2 antibody overnight at 4°C. The protein–antibody complex was then precipitated by incubating with 20 µl of protein G plus protein A agarose (Oncogene) for 1 h at 4°C and centrifuging at 2,000g at 4°C for 1 h. Both the precipitate and supernatant fractions were then analyzed by immunoblotting using the monoclonal B9 anti-human Bax antibody.

DNA Fragmentation Assay

Cells were resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 10 mM EDTA, 1% SDS, and 0.5 mg proteinase K, and incubated for 24 h at 37°C. A DNase-free RNase (Sigma) was then added into the cell lysate at a final concentration of 0.2 mg/ml, followed by an additional incubation at 37°C for 1 h. DNA was then precipitated by isopropanol, washed once with 75% ethanol, and dissolved in Tris-EDTA buffer (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA). Fifteen micrograms of DNA per sample was subjected to electrophoresis on 1.2% agarose containing 0.5 µg /ml of ethidium bromide.

TdT-Mediated dUTP Nick End Labeling Assay

TdT-Mediated dUTP Nick End Labeling (TUNEL) assay was performed with a Fluorescein-FragEL DNA Fragmentation Detection Kit (Oncogene Research Products), according to the manufacturer's instructions. Briefly, cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then in 80% ethanol overnight at −20°C. After that, cells were washed once with a buffer containing 20 mM Tris, pH 7.6, and 140 mM NaCl, permeabilized with proteinase K, and labeled with fluorescein-conjugated dNTPs and TdT enzyme, followed by analysis with flow cytometry.

RESULTS

Bax Is Cleaved Before the Apoptotic Execution by a Calpainlike Activity

We had reported that RB was internally cleaved by a caspaselike activity in the beginning of the apoptotic execution phase, associated with cleavage of PARP and the internucleosomal fragmentation of DNA [An and Dou, 1996; Fattman et al., 1997; An et al., 1998]. Most recently, one group reported that several hours after cleavage of PARP and RB as well as fragmentation of cellular DNA, the proapoptotic Bax protein was cleaved into a Bax/p18 fragment by a calpainlike activity [Wood et al., 1998; Wood and Newcomb, 1999]. We measured levels of Bax expression during apoptosis under our experimental conditions. We found that Bax cleavage occurred 3 h before the initiation of the apoptotic execution phase (Fig. 1). In this experiment, when human Jurkat T cells were treated with the anticancer agent etoposide/VP-16 (50 µM), the apoptotic execution phase began after 6 h, as demonstrated by processing/activation of caspase-3 (Fig. 1A, lanes 1–5), cleavage of PARP (Fig. 1B, lanes 1–5), and RB [An et al., 1998], and fragmentation of cellular DNA measured by both agarose gel electrophoresis (Fig. 1F, lanes 1–5) and TUNEL assay (Fig. 1G).

Levels of Bax protein expression were measured by Western blot analysis of the same cell extracts. A specific monoclonal B-9 antibody that was raised from a Bax fragment of amino acids 1–171 (representing all but the carboxyl terminal 21 amino acids) detected the full-length Bax protein (Bax/p21) in the untreated and treated cells (Fig. 1C). The B9 antibody also detected a band of 18 kDa (Bax/p18) and a band of ∼36 kDa (p36) in the drug-treated, but not untreated, cells at as early as 3 h (Fig. 1C, lanes 1–5). The p36 band most likely contains Bax/p18 (see the below figures and Discussion section). Another specific monoclonal 6A7 Bax antibody, which reacts with an epitope between amino acids 12 and 24 of Bax, detected only Bax/p21, but not Bax/p18 or p36 (Fig. 1D, lanes 1–5), indicating that Bax/p18 is an N-terminal cleaved form of Bax. Therefore, similar to the previous reports [Wood and Newcomb, 1999; Wood et al., 1998], we also found that Bax/p21 was cleaved at its N terminus, resulting in production of a p18 fragment. However, in contrast to the previous report [Wood and Newcomb, 1999], in which Bax cleavage occurred several hours after cleavage of PARP and RB as well as fragmentation of DNA (we refer to this as late Bax cleavage), we found that Bax was cleaved several hours be-
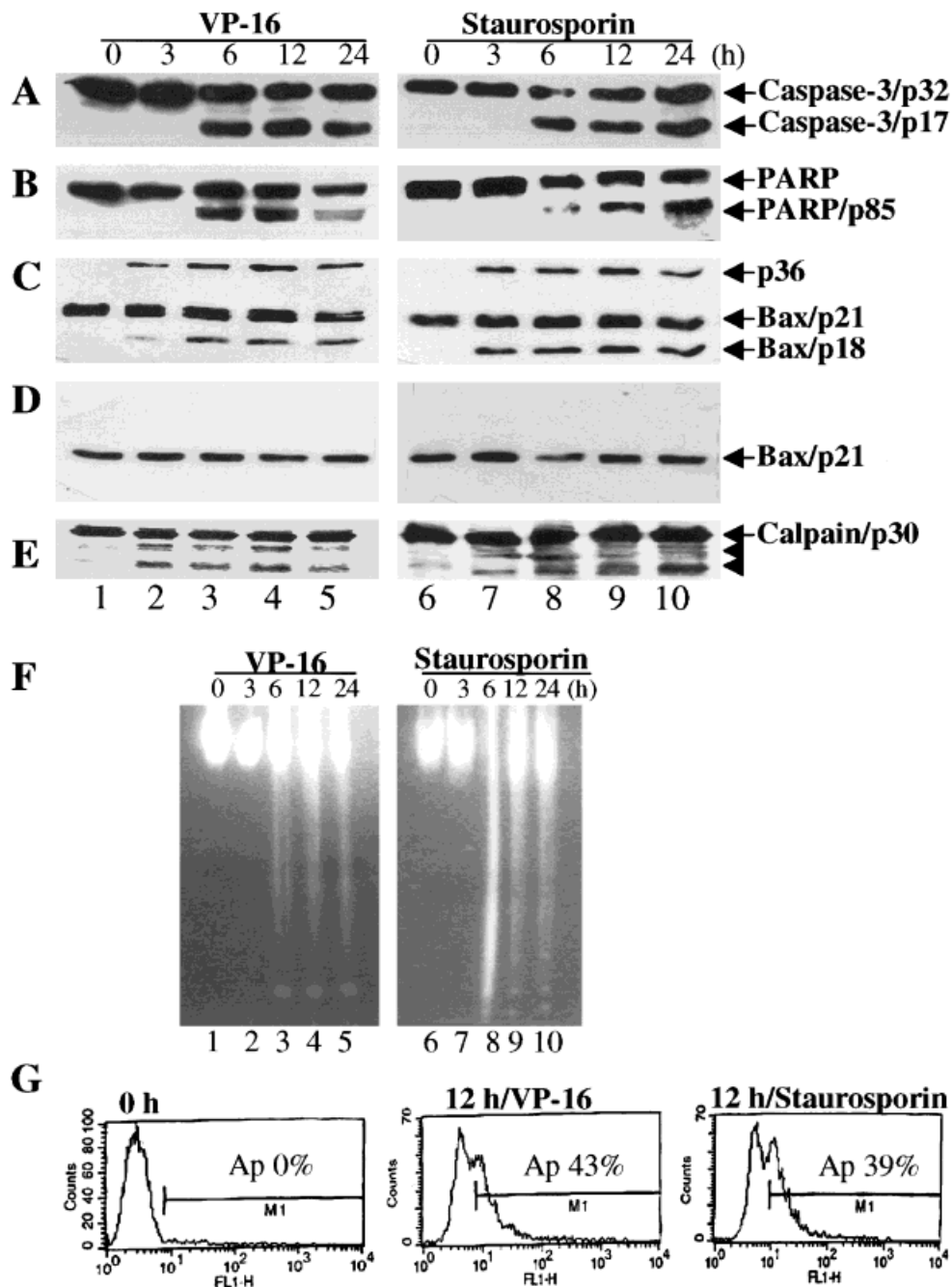
Fig. 1. Cleavage of Bax before initiation of apoptotic execution. Jurkat T cells were treated with 50 μM of VP-16 or 1 μM of stautosporin for the indicated hours. At each time point, cells were harvested and used for Western blotting (A–E), DNA fragmentation (F), and TdT-mediated dUTP nick end labeling (TUNEL) (G) assays. **A:** Caspase-3 processing and activation. Procaspase-3 (p32) and an active form of caspase-3 (p17) are indicated. **B:** Poly(ADP-ribose) polymerase (PARP) cleavage. The intact PARP (MW 116 kDa) and a PARP cleavage fragment (PARP/p85) are shown. **C:** The monoclonal B-9 anti-Bax antibody detects both full-length Bax (Bax/p21) and the cleaved form of Bax (Bax/p18). The p36 band is a Bax/p18-containing protein (see Discussion section). **D:** The monoclonal 6A7 anti-Bax antibody detects only the full-length Bax (Bax/p21). Note the absence of Bax/p18 and p36 bands. **E:** Autolysis/activation of the calpain 30-kDa subunit. The calpain 30-kDa subunit (p30) and at least two cleaved, active fragments (~28 and 22 kDa, respectively) are indicated. **F:** DNA fragmentation assay. DNA extracted from cells at each time point was subjected to agarose gel electrophoresis and visualized under ultraviolet light. **G:** TUNEL assay. The percentages of TUNEL-positive, apoptotic cell population (Ap) are indicated.
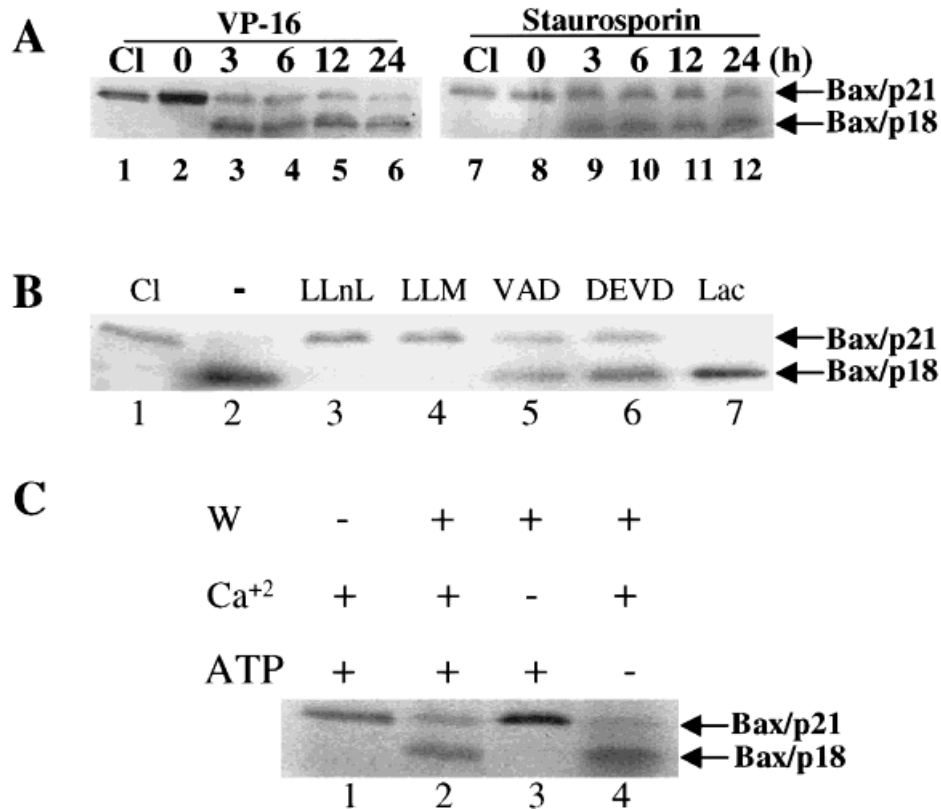
Fig. 2. Cell-free Bax cleavage activity. **A:** Detection of Bax cleavage activity in protein extracts of cells treated with VP-16 or staurosporin. [^{35}S]methionine-labeled Bax protein was incubated for 2 h with either buffer alone (as a control, Cl) or a whole cell lysate of Jurkat T cells untreated (0 h) or treated by VP-16 or staurosporin for the indicated hours, followed by gel electrophoresis and autoradiography. Both labeled full-length (Bax/p21) and the cleaved Bax (Bax/p18) are indicated. **B:** Sensitivity of the cell-free Bax cleavage activity to different chemical inhibitors. The ^{35}S-labeled Bax protein was incubated for 2 h with either buffer alone (Cl) or a whole lysate of Jurkat T cells pretreated with VP-16 for 12 h, in the absence ($-$) or presence of a protease inhibitor, including the calpain inhibitor-1 (LLnL, 10 μM), the calpain inhibitor-2 (LLM, 10 μM), the pan-caspase inhibitor Z-VAD-FMK (VAD, 20 μM), the caspase-3 specific inhibitor Ac-DEVD-CMK (DEVD, 20 μM), or the specific proteasome inhibitor β-lactone (Lac, 20 μM). **C:** Dependence of Bax cleavage activity on calcium. Cell-free Bax cleavage assay using a whole cell lysate (W) was performed as in (B), in the absence (lane 3, with 10 mM ATP) or presence of 5 mM Ca^{+2} (lane 2, with 10 mM ATP), or in the absence of ATP (lane 4, with 5 mM Ca^{+2}).

fore the initiation of the apoptotic execution (we refer to this as early Bax cleavage).

To provide additional evidence that Bax/p18 was a cleavage product of Bax/p21, we measured levels of Bax cleavage activity under cell-free conditions using an in vitro–translated, [^{35}S]-labeled Bax protein as substrate (Fig. 2A, lane 1). The Bax cleavage activity was absent in a whole protein extract prepared from exponentially growing Jurkat T cells, but was present in preparations from the cells that has been treated with VP-16 for 3 h or longer (Fig. 2A, lanes 2–6). The kinetics of cell-free Bax cleavage activity matched exactly that of Bax/p18 production in cells treated with VP-16 (compare Figs. 2A, lanes 2–6, to Fig. 1C, lanes 1–5).

The previously reported late Bax cleavage was mediated by an activated calpain activity [Wood et al., 1998]. We then investigated whether the early Bax cleavage was also mediated by a calpainlike activity. Because autolysis of the 30-kDa subunit of calpain is associated with its activation during apoptosis [Wood et al., 1998; Nath et al., 1996], we first measured kinetics of autolysis/activation of the calpain in the same Jurkat cells treated with VP-16. The autolysis of the calpain 30-kDa subunit, as demonstrated by appearance of two fragments ($\sim$28 kDa and $\sim$22 kDa), began at 3 h and continued at later time points (Fig. 1E, lanes 1–5), parallel to the kinetics of Bax cleavage in vivo (Fig. 1C, lanes 1–5) and in vitro (Fig. 2A, lanes 2–6).

To provide further evidence for calpain as Bax cleavage activity under our experimental conditions, we used specific inhibitors. The cell-free Bax cleavage activity in a whole cell extract was completely inhibited by 10 μM of the calpain inhibitor-1 (LLnL) or -2 (LLM), but not by 20 μM of the specific proteasome inhibitor clastolactacystin β-lactone (Fig. 2B, lanes 3, 4, 7 vs. 2), indicating that the early Bax cleavage activity is related to the calpain family. The process of in vitro Bax cleavage was partially inhibited by 20 μM of the general caspase inhibitor Z-VAD-FMK or the relatively specific caspase-3 inhibitor Ac-DEVD-CMK (Fig. 2B, lanes 5, 6 vs. 2), suggesting involvement of caspases in calpain activation (see Discussion section). The cell-free Bax cleavage activity was also blocked when Ca^{+2} was not added (Fig. 2C, lanes 3 vs. 2), demonstrating that it is a Ca^{+2}-dependent protease activity. However, the cell-free Bax cleavage process still occurred when ATP was not added (Fig. 2C, lane 4).

We then investigated whether the early Bax cleavage process was also inducible by a non-DNA damage agent. To do so, Jurkat T cells were treated with the kinase inhibitor staurosporin (1 μM) for up to 24 h. Again, processing/activation of caspase-3, cleavage of PARP, and fragmentation of DNA occurred at 6 h and later (Fig. 1A, B, F, lanes 6–10, and 1G). In comparison, Bax cleavage began at 3 h, which was detected by the monoclonal B9, but not 6A7, anti-Bax antibody (Fig. 1C,D, lanes 6–10). In addition, cell-free Bax cleavage activity was also observed when protein extracts were prepared from cells treated with staurosporin for 3 to 24 h, but not in an untreated cell preparation (Fig. 2A, lanes 7–12). Therefore, under our experimental conditions, before the initiation of apoptotic execution, both VP-16 and staurosporin activate a calpainlike enzyme, which in turn cleaves Bax into the Bax/p18 fragment.

Bax Cleavage Occurs in Mitochondria

The observation that the cleavage of Bax preceded activation of the effector caspase-3 (Fig. 1C vs. 1A) suggested the possible release of mitochondrial cytochrome c into the cytosol [Green and Reed, 1998; Adams and Cory, 1998; Gross et al., 1999]. To investigate this possibility, levels of cytochrome c were measured in both cytosolic and membrane-bound (enriched by mitochondria) fractions of the cells that had been treated with either VP-16 or staurosporin

for different hours, as described in Figure 1. No cytosolic cytochrome c was detectable in untreated cells, which appeared after 3 h treatment with either VP-16 or staurosporin, and further increased after a longer treatment (Fig. 3A). The increased levels of cytosolic cytochrome c were accompanied by decreased levels of the mitochondrial cytochrome c (Fig. 3B vs. 3A). The observed cytochrome c release from mitochondria to the cytosol was not an artifact, because we also observed constitutive levels of an ~40-kDa protein band in the cytosolic fractions (indicated by an arrowhead, Fig. 3A; the identity of this protein is unknown) and the mitochondrial cytochrome oxidase (COX) [Barrell et al., 1979] in the membrane-bound fractions (Fig. 3C). Importantly, release of cytochrome c was observed 3 h before processing/activation of caspase-3 (Figs. 3A vs. 1A).

The tight correlation between production of Bax/p18 and release of mitochondrial cytochrome c (Figs. 1C and 3A) suggested that Bax/p18 was probably a potent proapoptotic molecule with a cytochrome c-releasing activity. If so, Bax/p18 should be found in the mitochondrial fraction. To test this idea, both cytosolic and mitochondrial fractions of Jurkat T cells, which were either untreated or treated with VP-16 for 12 or 24 h, were subjected to Western blot analysis using the specific B9 anti-Bax antibody. In untreated cells, most of Bax/p21 was found in the cytosol fraction; during the drug treatment, the mitochondrial Bax/p21 level was increased (Fig. 3D), as demonstrated previously [Gross et al., 1999]. In contrast, both Bax/p18 and p36 proteins were detected only in the mitochondrial fraction of the drug-treated cells (Fig. 3D). Bcl-2 protein was only found in the mitochondrial fraction, and the mitochondrial Bcl-2 levels were decreased after a 24-h treatment with VP-16 (Fig. 3E).

Localization of Bax/p18, Bax/p21 and Bcl-2 in the mitochondrial membrane-enriched fraction (Fig. 3D,E) led us to examine whether they interacted with each other in the mitochondria during the process of VP-16-induced apoptosis. To do so, we performed a coupled immunoprecipitation–Western blot assay. Mitochondrial fractions were first prepared from Jurkat T cells that were either untreated or treated with VP-16 for 12 h, followed by preparation of Bcl-2 immunoprecipitates using a specific monoclonal antibody. The obtained Bcl-2 immunoprecipitates and the im-
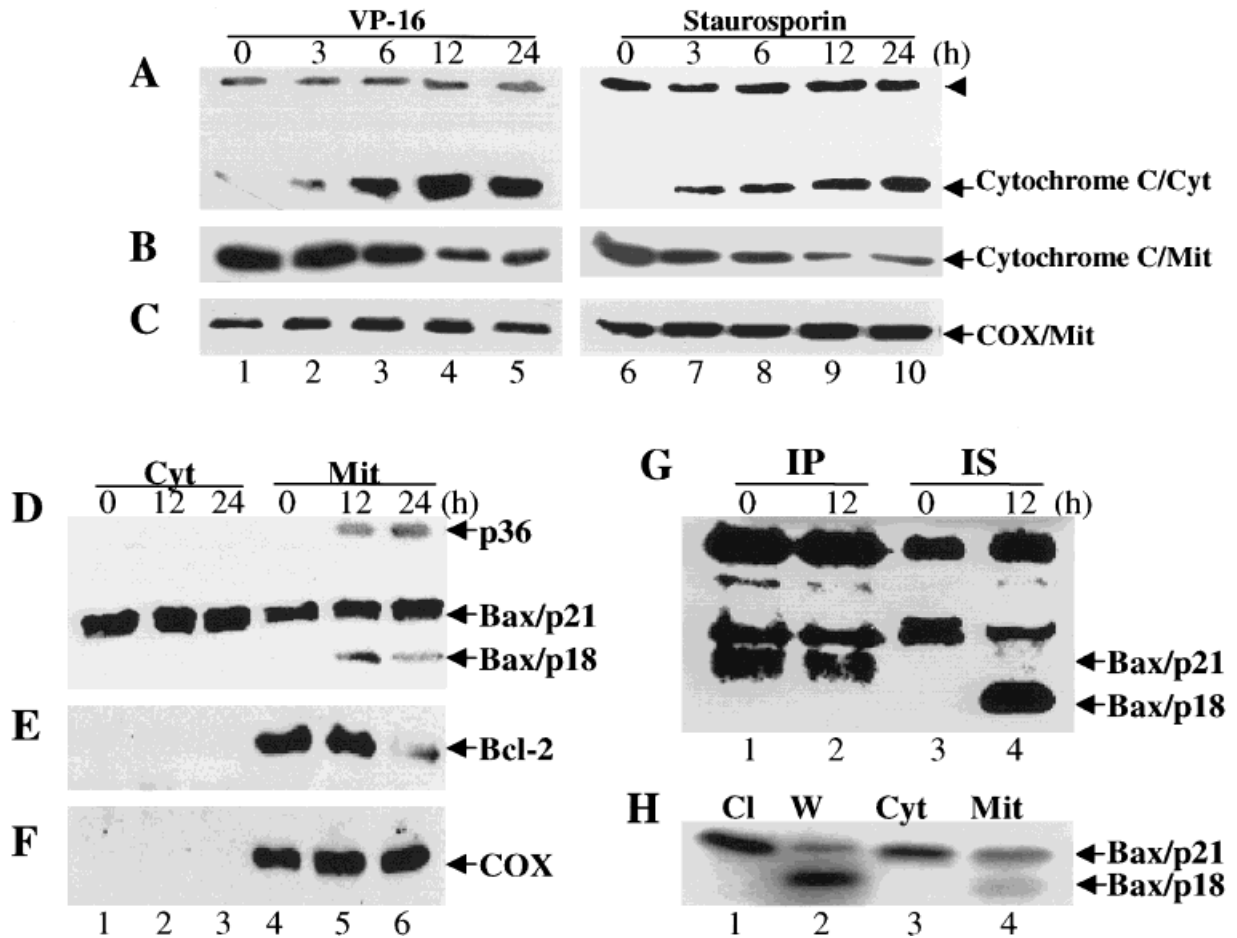
Fig. 3. Bax cleavage occurs in mitochondria. **A–C:** Cytochrome c release. Cytosol (Cyt) and mitochondria (Mit) fractions were prepared from Jurkat T cells treated with either 50 μM of VP-16 or 1 μM of stautosporin for the indicated hours, followed by Western blot assay using a specific antibody to cytochrome c (MW 17 kDa; A, B). A 40-kDa band, detected in the cytosol fractions and indicated by an arrowhead (A), was also shown as a loading control. The nature of this 40-kDa protein is unclear. The filter containing the mitochondrial fractions (B) was reblotted for the mitochondrial cytochrome oxidase subunit II (COX/Mit, MW 26 kDa), which served as a loading control (C). **D–F:** Localization of Bax/p18, Bax/p21, and Bcl-2. Cytosol (Cyt) and mitochondria (Mit) fractions were prepared from Jurkat T cells untreated (0 h) or treated with VP-16 for 12 or 24 h, followed by Western blotting with specific antibodies to Bax [the B9 antibody; (D)], Bcl-2 [MW 28 kDa; (E)], or COX (F). The positions of Bax/p21, Bax/p18, p36, Bcl-2, and COX are indicated. **G:** Bax/p18 does not interact with Bcl-2 in the mitochondrial fraction. The mitochondrial membrane-enriched fraction, prepared from Jurkat T cells either untreated (0 h) or treated for 12 h with VP-16, was incubated with a monoclonal anti-Bcl-2 antibody, followed by collecting both immunoprecipitate (IP) and the supernatant (IS) fractions, which were then used for Western blotting with the B-9 anti-Bax antibody. Both full-length (Bax/p21) and the cleaved (Bax/p18) Bax are indicated. Some immunoglobulin G chains are shown as a loading control. **H:** The Bax cleavage activity is present in the mitochondrial, but not the cytosol, fraction. [^{35}S]methionine-labeled Bax protein was incubated for 2 h with either buffer alone (Cl), a whole cell lysate (W), cytosol (Cyt), or mitochondrial (Mit) fraction of Jurkat T cells treated with VP-16 for 12 h, followed by analysis of Bax cleavage product as described in the legend of Figure 2.

munodepleted supernatant fractions were then immunoblotted with the B9 anti-Bax antibody (Fig. 3G). All the Bax/p21 protein was coimmunoprecipitated by the anti-Bcl-2 antibody from both untreated and drug-treated cells (Fig. 3G), indicating interaction of Bax/p21 and Bcl-2 in mitochondria under both nonapoptotic and apoptotic conditions. In contrast, all the

Bax/p18 fragment was detected only in the anti-Bcl-2-depleted supernatant of the drug-treated cells (Fig. 3G), demonstrating that Bax/p18, although it was also present in mitochondria, did not interact with Bcl-2 protein.

The mitochondrial localization of Bax/p18 could be caused by either cleavage of the mitochondrial Bax/p21 by a colocalized calpain ac-
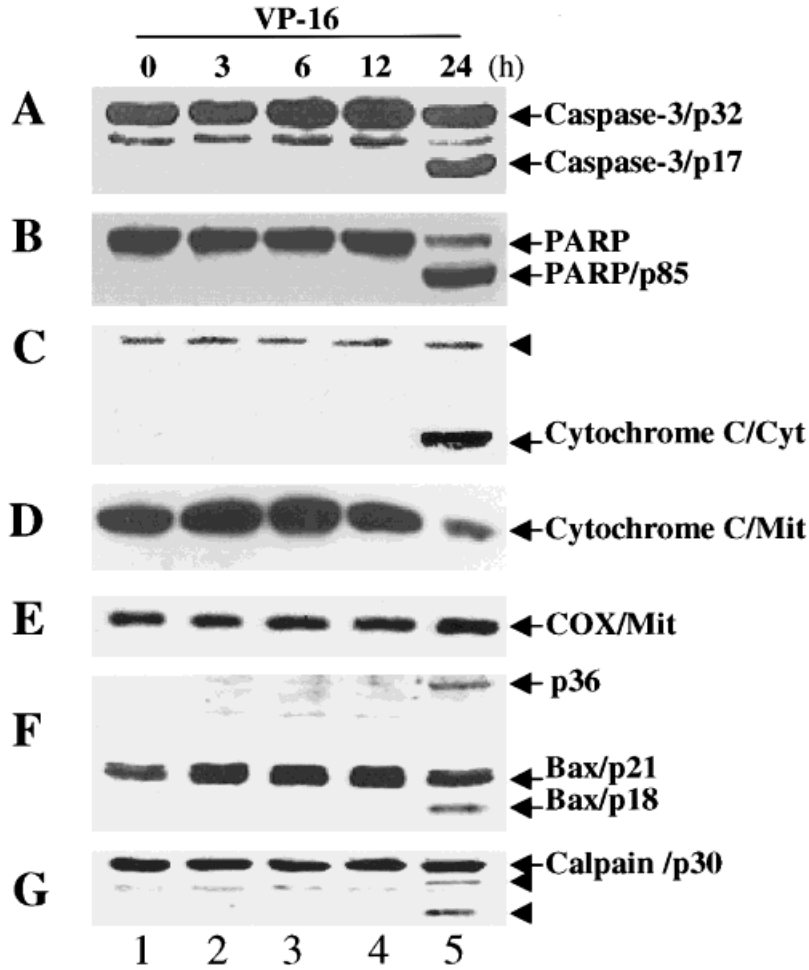
Fig. 4. Inhibition of Bax cleavage by the specific calpain inhibitor calpeptin is associated with inhibition of cytochrome c release and apoptosis execution. Jurkat T cells were pretreated with 10 μM of calpeptin for 1 h, followed by coincubation with 50 μM of VP-16 for up to 24 h, as indicated. At each time point, the cells were collected and used for measurement of caspase-3 processing/activation (**A**), poly(ADP-ribose) polymerase (PARP) cleavage (**B**), cytochrome c release (**C–E**), Bax cleavage [with B9 antibody; (**F**)], and calpain 30-kDa subunit autolysis (**G**), as described in the legends of Figures 1 and 3.

tivity or cleavage by calpain of the cytosolic Bax/p21, followed by translocation of the resultant Bax/p18 to mitochondria. To distinguish these two possibilities, cell-free Bax cleavage assay was performed using a mitochondrial, cytosol, or whole cell preparation from VP-16-treated Jurkat T cells. The labeled Bax protein was cleaved by the mitochondrial, but not the cytosolic, fraction (Fig. 3H, lanes 4 vs. 3), indicating the presence of early Bax cleavage activity in the mitochondria of cells treated with VP-16. However, more labeled Bax protein was cleaved by the whole cell extract than the mitochondrial fraction (Fig. 3H, lanes 2 vs. 4), suggesting that the mitochondrial Bax cleavage activity could be increased by some cytosolic factor(s) of drug-treated cells (see Discussion section). These data suggest that an activated mitochondrial calpain enzyme is probably responsible for cleavage of Bax/p21 into Bax/p18 before release of cytochome c and activation of caspase-3.

Pretreatment with the Specific Calpain Inhibitor Calpeptin Delays VP-16-Induced Calpain Autolysis/Activation, Bax Cleavage, Cytochrome C Release, Caspase-3 Processing/Activation, and Apoptosis

Activation of the calpainlike activity and subsequent cleavage of Bax preceding cytochrome c-associated apoptosis induction (Figs. 1, 3) suggested a critical role of Bax/p18 in committing a cell to releasing cytochrome c and undergoing apoptosis. If so, a specific calpain inhibitor should be able to prevent Bax from being cleaved and consequently inhibit the mitochondrial cytochrome c release and apoptosis. We then tested this idea by pretreating Jurkat T cells for 1 h with 10 μM of a specific calpain inhibitor calpeptin [Medhi, 1991], which itself did not induce apoptosis (Fig. 4, lane 1) [Wood et al., 1998]. After that, 50 μM of VP-16 was added (in the presence of calpeptin) and the cells were then incubated for up to

24 h. The pre- and cotreatment with calpeptin delayed the VP-16-induced caspase-3 processing and PARP cleavage from 6 to 24 h (Figs. 4 vs. 1, A and B, lanes 1–5). The delayed caspase-3 activation was likely caused by a delay in release of cytochrome c from mitochondria to the cytosol (Fig. 4C–E, vs. Fig. 3A–C, lanes 1–5). In addition, the delayed cytochrome c release was also associated with a delay in the process of Bax cleavage (Figs. 4F vs. 1C, lanes 1–5). Consistent with the delay in Bax cleavage, autolysis of the 30-kDa small subunit of calpain was also delayed from 3 h to 24 h (Figs. 4G vs. 1E, lanes 1–5). These data suggest that autolysis of calpain is required for its activation and that the activated calpain is required for Bax cleavage. Taken together, our data suggest that an active mitochondrial calpain enzyme is likely responsible for the early Bax cleavage and that the produced Bax/p18 mediates the cytochrome c–regulated apoptotic process.

Overexpression of Bax/p18 in Mitochondria Is Sufficient to Induce Cytochrome C Release and Apoptotic Cell Death

We hypothesized that our early Bax cleavage activity was similar or identical to the late Bax cleavage activity reported previously [Wood et al., 1998], because both activities were dependent on calcium (Fig. 2C), inhibitable by specific calpain inhibitors in vitro and in vivo (Figs. 2B and 4), and associated with autolysis of the 30-kDa subunit of calpain (Fig. 1E) [Wood et al., 1998]. If so, the cleavage site on Bax by the early activated calpain activity should be the same as the site cleaved by the late calpain activity. The Bax cleavage site for the late calpain activity was identified to be around amino acids 30–33 (FIQD) of Bax [Wood et al., 1998]. We then used PCR techniques and cloned the cDNA sequence corresponding to a fragment of amino acids 33–192 of Bax (Fig. 5A). The Bax/p18 cDNA was then subcloned into a pcDNA3.0 vector. To directly test the idea that the cloned Bax/p18 is a mediator of cytochrome c release and apoptosis, Jurkat T cells (control in Fig. 5B, lane 1) were transiently transfected with either pcDNA3.0 vector alone or pcDNA3.0 vector containing Bax/p18 or Bax/p21 cDNA. After a 48-h transfection, cells were harvested for measurement of Bax expression, Bax localization, cytochrome c release, and apoptosis (Fig. 5). Western blotting using the monoclonal B9 anti-Bax antibody revealed high levels of Bax/p18 and p36 proteins in the Bax/p18 cDNA-transfected cells (Fig. 5B, lane 3), demonstrating that p36 contains Bax/p18. High levels of Bax/p21 protein were observed in the full-length Bax cDNA-transfected cells (Fig. 5B, lane 4). Overexpression of Bax/p21, but not Bax/p18, was also detected by the monoclonal 6A7 Bax antibody (Fig. 5C, lanes 1–4), consistent with the fact that the transfected Bax/p18 did not contain the N-terminal sequence.

To examine the localization of the transfected Bax/p18 protein, both cytosolic and mitochondrial fractions were prepared from Jurkat T cells transiently transfected with either vector alone or Bax/p18 cDNA, and analyzed by Western blotting using the B9 Bax antibody. All the transfected Bax/p18 protein was found in the mitochondrial, but not the cytosolic, fraction (Fig. 5D, lanes 4 vs. 2). The p36 band was also found only in the mitochondrial fraction of the Bax/p18 cDNA transfected cells (Fig. 5D, lanes 4 vs. 2).

To directly test whether Bax/p18 has the cytochrome c–releasing ability, levels of both cytosolic and mitochondrial cytochrome c were measured in the transfected Jurkat T cells. Release of cytochrome c was induced in the cells transfected with Bax/p18 cDNA, as demonstrated by an increased level of the cytosolic cytochrome c and a decreased level of the mitochondrial cytochrome c (Fig. 5, F–H, lanes 1–3). In contrast, cytochrome c release was not observed in the cells transfected with the Bax/p21 cDNA (Fig. 5F,G, lanes 4 vs. 2) (see Discussion section). Furthermore, caspase-3 processing/activation (data not shown), PARP cleavage, and DNA fragmentation (Fig. 5I,J, lanes 1–4) were also detected only in the cells transfected with Bax/p18, but not Bax/p21, cDNA.

To confirm the cytochrome c–releasing and apoptosis-inducing ability of Bax/p18, we transiently transfected another cell line, human breast cancer MCF-7, with Bax/p18 cDNA, using the vector and Bax/p21 cDNA plasmid as controls (Fig. 5B–J, lanes 5–8). Again, high levels of Bax/p18 and p36 were found in the Bax/p18 cDNA-transfected MCF-7 cells, and high levels of full-length Bax protein were in the Bax cDNA-transfected MCF-7 cells (Fig. 5B,C, lanes 5–8). All the overexpressed Bax/p18 and p36 in MCF-7 cells were again found
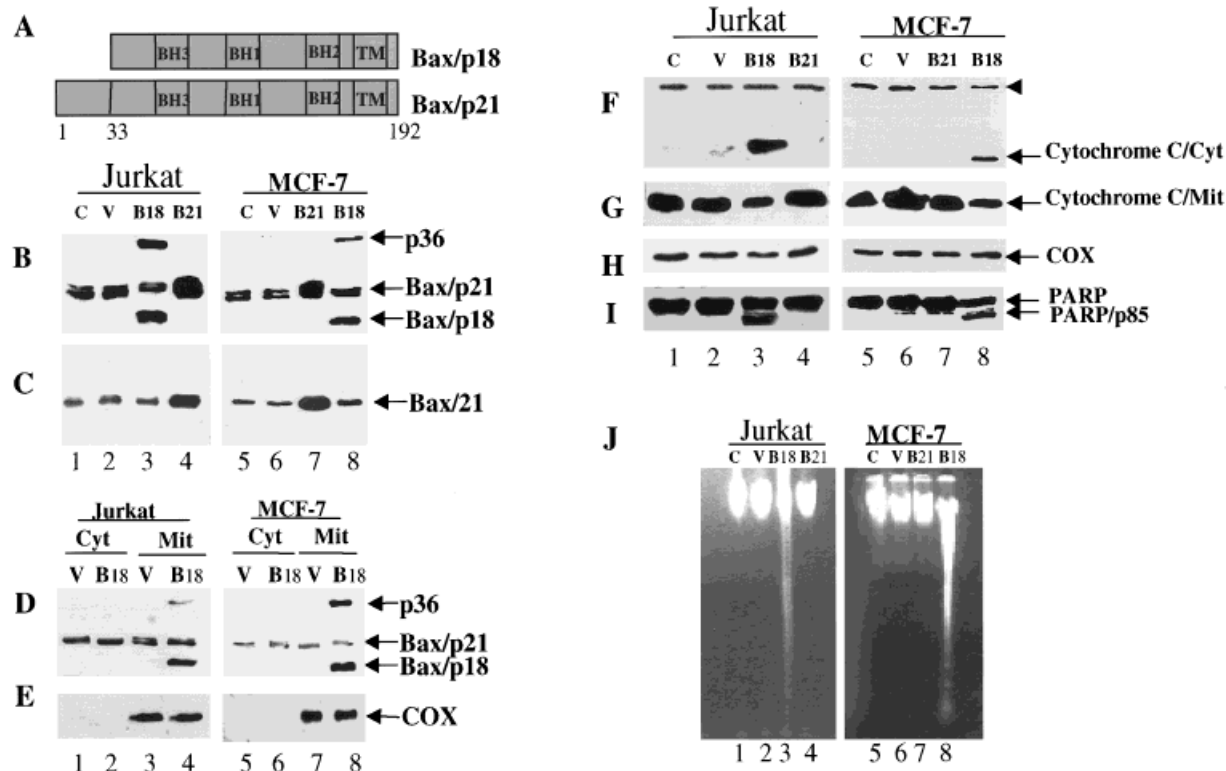
Fig. 5. Overexpression of Bax/p18 in mitochondria induces cytochrome c release and apoptotic cell death. **A:** A schematic diagram of Bax/p18 and full-length Bax protein encoded by the corresponding cDNAs. The cloned Bax/p18 consists of amino acids 33–192 of Bax. The BH1, BH2, BH3, and the transmembrane (TM) domains are indicated. **B–J:** Jurkat or MCF-7 cells (C for control) were transiently transfected with pcDNA3 vector alone (V) or pcDNA3 containing Bax/p18 (B18) or Bax-α cDNA (B21). After transfection, cells were used for measurement of Bax expression [(B), (C), with B9 and 6A7 antibodies, respectively] and localization [(D), (E), with B9 anti-Bax and anti-COX antibodies, respectively], cytochrome c release (F–H), poly(ADP-ribose) polymerase (PARP) cleavage (I), and DNA fragmentation (J), as described in the legends of Figures 1 and 3.

in the mitochondrial preparation (Fig. 5D,E, lanes 5–8). The mitochondrial localization of Bax/p18 in MCF-7 cells was associated with release of cytochrome c, cleavage of PARP, and fragmentation of DNA (Fig. 5F–J, lanes 5–8). Overexpression of Bax/p21 in MCF-7 cells again did not induce cytochrome c–associated apoptotic events (Fig. 5F–J, lanes 7 vs. 6). Therefore, transfection of Bax/p18 cDNA in both Jurkat and MCF-7 cells targeted Bax/p18 protein to mitochondria, which was sufficient to induce cytochrome c release and subsequent apoptotic cell death. From these data, we conclude that Bax/p18 has the cytochrome c–releasing ability.

Bax/p18-Induced Apoptosis Cannot Be Blocked by Overexpression of Bcl-2

The lack of interaction between Bax/p18 and Bcl-2 proteins in the mitochondrial fraction un-

der apoptotic conditions (Fig. 3G) suggested that Bax/p18-induced cytochrome c release and apoptotic cell death was independent of Bcl-2. If so, Bcl-2- and the vector-overexpressing cells should be equally sensitive to apoptosis induction by Bax/p18 transfection. To test this hypothesis, Jurkat T cells overexpressing Bcl-2 or vector alone (Neo) (Fig. 6A) were transiently transfected with pcDNA vector or pcDNA vector containing Bax/p18 cDNA, followed by measurement of Bax expression, Bax localization, cytochrome c release, and apoptosis induction. High levels of Bax/p18 and p36 proteins were found in Bcl-2-overexpressing cells transfected with the Bax/p18 cDNA, which were comparable to that in the Bax/p18-transfected Neo cells (Fig. 6B, lanes 6 vs. 3). Consistent with that, similar high levels of cytosolic cytochrome c were observed in both Bcl-2 and Neo cell lines transfected with the Bax/p18 cDNA, but not
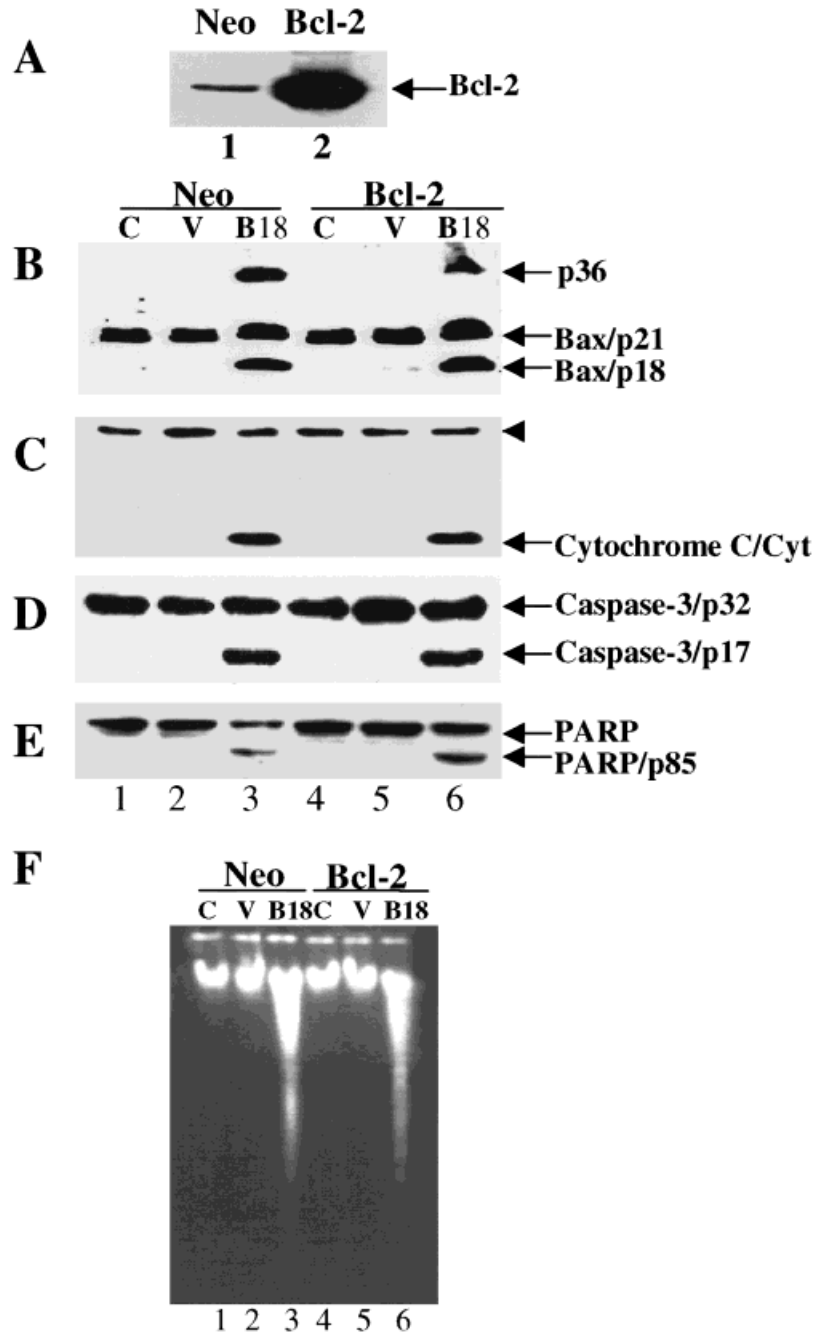
Fig. 6. Bax/p18-mediated apoptosis is not blocked by overexpression of Bcl-2. **A:** Bcl-2 levels in exponentially growing Neo and Bcl-2 cells. **B–F:** Both Neo and Bcl-2 cells (C for control) were transiently transfected with pcDNA3 vector (V) or pcDNA3 vector containing Bax/p18 cDNA (B18), followed by measurement of Bax expression [(B), with B9 antibody], cytochrome c release (C), caspase 3 processing/activation (D), poly(ADP-ribose) polymerase (PARP) cleavage (E), and DNA fragmentation (F), as described in Figures 1 and 3.

the pcDNA vector (Fig. 6C, lanes 1-6). Furthermore, similar levels of induced apoptosis were also obtained in both Bcl-2 and Neo cell lines transfected with the Bax/p18 cDNA but not the pcDNA vector, as demonstrated by processing of caspase-3, cleavage of PARP, and fragmentation of DNA (Fig. 6D–F, lanes 1–6). These data demonstrate that Bcl-2 is unable to block Bax/p18-mediated cytochrome c release and apoptosis.

Overexpression of Bcl-2 Protein Delays VP-16-Induced Calpain Autolysis, Bax Cleavage, Cytochrome C Release, and Apoptosis Induction

Although Bcl-2 overexpression did not block Bax/p18-mediated apoptosis (Fig. 6), Bcl-2 had been shown to repress the cellular apoptosis process triggered by a diverse array of stimuli including chemotherapeutic anticancer drugs
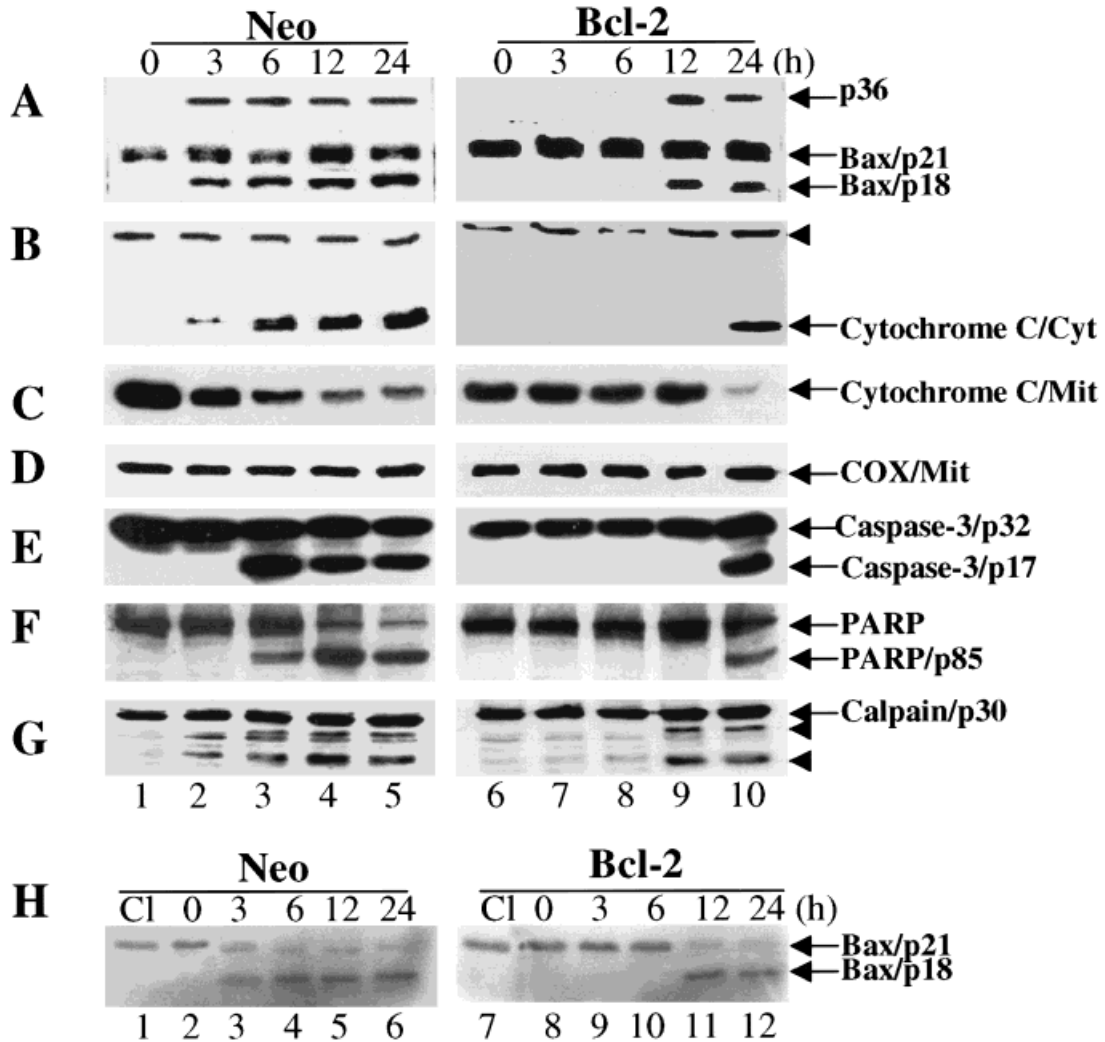
Fig. 7. Overexpression of Bcl-2 delays VP-16-induced calpain autolysis, Bax cleavage, cytochrome c release, and apoptosis induction. Neo and Bcl-2 cells were treated with 50 µM of VP-16 for indicated hours, followed by measurement of Bax expression (**A**, with B9 antibody), cytochrome c release (**B–D**), caspase-3 processing/activation (**E**), poly(ADP-ribose) polymerase (PARP) cleavage (**F**), and calpain 30-kDa subunit autolysis (**G**), as described in Figures 1 and 3. An aliquot of the whole cell extract at each time point was also used for cell-free Bax cleavage assay (**H**), as described in the legend of Figure 2.

[Green and Reed, 1998; Adams and Cory, 1998; Gross et al., 1999]. We reasoned that if production of Bax/p18 was important for apoptosis induced by VP-16 (Fig. 1), the drug-induced Bax cleavage process should be inhibited in cells overexpressing Bcl-2. To test this idea, both Bcl-2 and Neo cells were treated with 50 µM of VP-16 for 3, 6, 12, or 24 h. At each time point, aliquots of the cells were used to assay expression of Bax, release of cytochrome c, and induction of apoptosis. Kinetics of apoptosis-associated events induced in Neo cells was virtually identical to that in parental Jurkat cells

(Fig. 7 vs. Figs. 1 and 3). In both Neo and parental cell lines treated with VP-16, the appearance of Bax/p18 and p36 bands was detected at as early as 3 h (Fig. 7A vs. Fig. 1C, lanes 1–5), associated with increased levels of cytosolic cytochrome c and decreased levels of mitochondrial cytochrome c (Fig. 7B–D vs. Fig. 3A–C, lanes 1–5). In addition, after 6 h or longer treatment of both Neo and Jurkat cell lines, apoptosis was induced, as evident by processing of caspase-3 and cleavage of PARP (Fig. 7E,F vs. Fig. 1A,B, lanes 1–5). However, all these VP-16-induced events were delayed in

Bcl-2-overexpressing cells. Appearance of Bax/ p18 and p36 bands was observed in Bcl-2 cells at 12 and 24 h of VP-16 treatment (Fig. 7A, lanes 6–10), indicating a 9-h delay (Fig. 7A, lanes 9 vs. 2). Consistent with that, release of cytochrome c was detected after 12 h in Bcl-2 cells treated with VP-16 (Fig. 7B–D, lanes 6–10). Associated with the delayed cytochrome c release, both caspase-3 processing and PARP cleavage were observed only after 24 h VP-16 treatment of Bcl-2 cells (Fig. 7E,F, lanes 6–10). These data suggest that Bcl-2 overexpression delays VP-16-induced cleavage of Bax/p21 into Bax/p18, and subsequently inhibits cytochrome c-dependent apoptosis.

To investigate whether the delayed events observed in Bcl-2-overexpressing cells were caused by inhibition of calpain activation, we measured levels of calpain autoproteolysis and cell-free Bax cleavage activity in the Bcl-2 and Neo cells that had been treated with VP-16 for different hours. Overexpression of Bcl-2 delayed the autolysis process of calpain 30-kDa subunit from 3 to 12 h (Fig. 7G, lanes 9 vs. 2). In addition, detection of cell-free Bax cleavage activity was also delayed by 9 h in Bcl-2-overexpressing vs. Neo cells (Fig. 7H, lanes 11 vs. 3). The delayed calpain autolysis and Bax cleavage activity in Bcl-2 vs. Neo cells were paralleled to the delayed Bax cleavage process measured in vivo (Fig. 7G,H vs. 7A). Therefore, Bcl-2 overexpression delayed VP-16-induced calpain activation and subsequently Bax cleavage. Because the levels of overexpressed Bcl-2 protein remained unchanged during the VP-16 treatment (data not shown), it is possible that activation of the Bax cleavage enzyme calpain, although inhibitable by Bcl-2 overexpression, can also be induced by VP-16 via a Bcl-2-independent pathway. Taken together, our data further support the argument that Bax/ p18, generated by an active calpain enzyme, promotes cytochrome c release and induces cell death.

DISCUSSION

It has been suggested that Bax protein exerts at least part of its proapoptotic activity by triggering cytochrome c release from mitochondria [Green and Reed, 1998; Adams and Cory, 1998; Gross et al., 1999]. Although the molecular mechanisms by which Bax stimulates cytochrome c efflux are still unknown, several apoptosis-associated events occurring at the

level of Bax protein have been described. These events include translocation to mitochondria [Hsu et al., 1997; Wolter et al., 1997], change in conformation [Desagher et al., 1999], oligomerization [Goping et al., 1998; Gross et al., 1998], membrane integration [Goping et al., 1998; Gross et al., 1998], and cooperation in some of the above processes with other proteins such as Bid [Eskes et al., 2000; Desagher et al., 1999]. In the current study, we have reported an additional novel mechanism by which an active calpain enzyme cleaves Bax at its N-terminus, producing a potent proapoptotic Bax/p18 fragment that in turn induces cytochrome c release and drives programmed cell death. Specifically, we have reported the following new findings. First, calpain-mediated Bax cleavage occurred in a very early stage of VP-16- or staurosporin-induced apoptosis, which was associated with cytochrome c release but preceded caspase activation and the execution phase of apoptotic cell death (Figs. 1 and 3). Second, the following unique properties of the generated Bax/p18 fragment distinguished itself from the full-length Bax /p21. All the Bax/p18, but only a portion of Bax/p21, were found in the mitochondrial membrane-enriched fraction of drug-treated cells (Fig. 3D). Bax/p18, in contrast to the mitochondrial Bax/p21, did not interact with the antiapoptotic Bcl-2 protein (Fig. 3G). Third, treatment of cells with the specific calpain inhibitor calpeptin inhibited calpain autolysis/activation and Bax cleavage, and subsequently delayed the downstream events, including cytochrome c release, caspase activation, and apoptotic execution (Fig. 4). Fourth, we cloned the Bax/p18 cDNA and transfected it to multiple human cancer cell lines. The overexpressed Bax/p18 protein was found in the mitochondrial fraction, which was accompanied by release of cytochrome c and induction of apoptosis (Figs. 5 and 6), demonstrating that Bax/p18 has the cytochrome c–releasing ability. Fifth, overexpression of Bcl-2 did not block Bax/p18-induced apoptosis (Fig. 6). Finally, Bcl-2 overexpression inhibited drug-induced calpain autolysis/activation, Bax cleavage, and cytochrome c–regulated apoptosis (Fig. 7), indicating that at least one of the molecular mechanisms by which Bcl-2 inhibits apoptosis is its ability to inhibit the activation of calpain-mediated Bax cleavage activity.

All the results presented in this study support the argument that Bax/p18 is a potent

proapoptotic molecule that mediates cell death through release of mitochondrial cytochrome c into the cytosol in a Bcl-2 independent manner. Indeed, appearance of Bax/p18 was always found to be associated with cytochrome c release and before caspase-3 activation in Jurkat, MCF-7, and Neo cells treated with either VP-16 or staurosporin (Figs. 1, 3, and 7). In contrast, inhibition of drug-induced Bax cleavage by either calpeptin pretreatment or Bcl-2 overexpression resulted in inhibition of cytochrome c release and apoptotic cell death (Figs. 4 and 7). In addition, overexpression of Bax/p18 cDNA in multiple cancer cell lines was sufficient to trigger release of cytochrome c and induce apoptosis, which was not prevented by overexpression of Bcl-2 protein (Figs. 5 and 6). Furthermore, Bax/p18, produced by either drug treatment or transfection, was found in the mitochondrial membrane-enriched, but not the cytosol, fraction (Figs. 3, 5). Unlike the full-length Bax, the Bax/p18 did not interact with Bcl-2 protein in the mitochondrial fraction (Fig. 3G). Our data are consistent with an early report that deletion of the N-terminal 20 amino acids of Bax enabled its targeting to mitochondria in vitro [Goping et al., 1998], although such a Bax mutation has never been reported in vivo. In contrast to that, Bax/p18 can be generated in a cell after apoptotic death program is triggered (Figs. 1 and 7).

Our results did not rule out the possibility that dimerization or a posttranslational modification of Bax/p18 is essential for its proapoptotic function. Kinetically, whenever Bax/p18 was generated (i.e., by either VP-16 or staurosporin treatment), a p36 band was observed (Figs. 1, 7), whereas whenever production of Bax/p18 was inhibited or delayed (i.e., by calpeptin treatment or Bcl-2 overexpression), appearance of p36 was undetected or delayed (Figs. 4, 7). In addition, both Bax/p18 and p36 proteins were found in the mitochondrial membrane-enriched, but not the cytosol, fraction of drug-treated Jurkat cells (Fig. 3). Most importantly, transfection of different cell lines with Bax/p18, but not Bax/p21, cDNA resulted in generation of p36 band (Figs. 5, 6), and both overexpressed Bax/p18 and p36 proteins were found in the mitochondrial, but not cytosol, fraction of these cells (Fig. 5D). Only under certain in vitro conditions, detection of Bax/p18 was uncoupled from that of p36. For example, in the anti-Bcl-2-depleted supernatant frac-

tion, while an abundant band of Bax/p18 was observed, p36 was not apparent (Fig. 3G), suggesting dissociation of p36 into Bax/p18 during the immunoprecipitation process. In addition, when a labeled Bax/p18 was generated by in vitro incubation with a protein extract of cells treated with VP-16 or staurosporin, no labeled p36 was detected (data not shown). Although we can conclude that p36 is a Bax/p18-containing protein, the nature of p36 remains to be studied.

The early Bax cleavage activity, detected under our experimental conditions (Figs. 1, 7), is a calpainlike activity, which is supported by both in vitro and in vivo evidence: 1) The process of Bax cleavage under cell-free conditions was blocked by addition of calpain inhibitors LLM or LLnL, but not the proteasome inhibitor β-lactone (Fig. 2B). 2) The cell-free Bax cleavage activity is dependent of Ca^{+2} (Fig. 2C). 3) Pretreatment of cells with the specific calpain inhibitor calpeptin delayed the Bax cleavage process (Fig. 4). The delayed, rather than a complete, inhibition by calpeptin (Fig. 4) is probably caused by decreased levels of calpeptin by its metabolism (Fig. 4G, lane 5) because addition of fresh calpeptin into these cells at 18 h after VP-16 treatment resulted in a complete inhibition of PARP cleavage at 24 h (data not shown).

Previously, a late Bax cleavage activity, which was detected several hours after DNA fragmentation, was reported to be a calpainlike, but not caspaselike, activity [Wood and Newcomb, 1999; Wood et al., 1998]. Similar to the late Bax cleavage activity, the early Bax cleavage activity was also dependent on calcium (Fig. 2C), inhibitable by specific calpain inhibitors in vitro and in vivo (Figs. 2B and 4), and associated with autolysis/activation of the 30-kDa subunit of calpain (Fig. 1E). The Bax cleavage site for the late calpain activity was identified to be around amino acids $^{30}FIQD^{33}$ of Bax [Wood et al., 1998]. We reasoned that if both late and early Bax cleavage activities were similar or identical, their cleavage sites on Bax should be the same. If so, the cloned Bax/p18 fragment should have the cytochrome c–releasing activity. To examine this hypothesis, we cloned the Bax/p18 cDNA based on the reported Bax cleavage site [Wood and Newcomb, 1999], and transfected it to several human cancer cell lines. Indeed, overexpression of Bax/p18, which was accumulated in the mito-

chondrial fraction, was sufficient to trigger cytochrome c release and initiate apoptotic execution (Figs. 5 and 6). These data suggest that our cloned Bax/p18 cDNA encodes a Bax fragment that is identical, or very similar, to the one produced at the early stage of the apoptotic process (Fig. 1C).

We also found that under our cell-free conditions, the Bax cleavage activity was absent in a protein extract of growing tumor cells, but present in a preparation of cells treated with an apoptotic stimulus (Figs. 2A and 7H). In addition, the Bax cleavage activity appears to be located in mitochondria because the mitochondrial membrane-enriched, but not the cytosol, fraction was able to cleave a labeled Bax/p21 into Bax/p18 (Fig. 2C). However, some cytosolic factors may be required for upregulation of Bax cleavage activity because a whole cell extract cleaved more labeled Bax protein than a mitochondrial fraction (Fig. 2C). These cytosolic factors might regulate either conformation of Bax or levels of calpain and/or calpain inhibitors. Addition of a caspase inhibitor partially inhibited the cell-free Bax cleavage activity (Fig. 2B), suggesting that caspases positively regulate the process of Bax cleavage. Consistent with that, it has been reported that caspase-3 cleaves the calpain inhibitor protein calpastatin, leading to activation of calpain [Wang et al., 1998; Porn-Ares et al., 1998]. In addition, overexpression of Bcl-2 delayed activation of calpain, the Bax cleavage enzyme (Fig. 7G and H), indicating that Bcl-2 negatively regulates the Bax cleavage process. The delayed, rather than a complete, inhibition by Bcl-2 (Fig. 7), together with the observation that levels of overexpressed Bcl-2 protein were unchanged during the VP-16 treatment (data not shown), suggests existence of another pathway(s) for activation of Bax cleavage enzyme that is VP-16-inducable but Bcl-2-independent. Finally, it should also be noted that some cytosolic factors are required for Bax translocation [Nmura et al., 1999] and that cytosolic Bax is required for in vitro cytochrome c release [Schuler et al., 2000].

There are several differences between our experimental conditions under which the early Bax cleavage was observed (Figs. 1 and 7) and the conditions Wood and Newcomb used for detection of the late Bax cleavage [Wood and Newcomb, 1999]. First, different cell lines were used. We used Jurkat T and MCF-7 cells, whereas Wood and Newcomb used HL-60 cells [Wood and Newcomb, 1999]. Second, different apoptosis stimuli were used. In our experiments, we administered VP-16 and staurosporin, whereas Wood and Newcomb used a topoisomerase I inhibitor 9-amino-20(S)-camptothecin [Wood and Newcomb, 1999]. Third, different Bax antibodies were used to detect the appearance of Bax/p18. We used the specific monoclonal B-9 antibody that was raised from a Bax fragment of amino acids 1–171 (representing all but the carboxyl terminal 21 amino acids). The previous researchers used a RAb Bax antibody that was raised to epitope encoded by exon 3 (amino acids 43–61) [Wood et al., 1998]. Fourth, different specificity of cell-free Bax cleavage activity was observed. We detected the Bax cleavage activity only in the protein extracts of cells treated with VP-16 or staurosporin, but not in a preparation of untreated cells (Figs. 2A and 7H). In contrast, the previous researchers observed the Bax cleavage activity even in an untreated cell preparation [Wood et al., 1998]. Finally and most importantly, different results were obtained from experiments using the specific calpain inhibitor calpeptin. We found that calpeptin treatment delayed drug-induced calpain autolysis, Bax cleavage, cytochrome c release, caspase-3 activation, and PARP cleavage (Fig. 4). In contrast, Wood and Newcomb reported that calpeptin inhibited only drug-induced calpain autolysis and Bax cleavage, but not PARP cleavage and DNA fragmentation [Wood and Newcomb, 1999]. Therefore, it is possible that the early Bax cleavage plays a causative role in apoptosis, whereas the late Bax cleavage is a result of caspase activation [Wood and Newcomb, 1999].

It is unclear why the Bax cleavage enzyme, calpain, is activated early under our experimental conditions, but late in conditions used by Wood and Newcomb [Wood and Newcomb, 1999]. This difference may not be caused by different antibodies (because both antibodies can detect apoptosis-specific Bax cleavage in vivo) or drugs (because both VP-16 and the camptothecin analogue work via DNA damage-induced signal transduction pathway). The difference is probably caused by the different cell systems used. It is possible that HL-60 cells have high levels of a calpain inhibitor such as calpastatin, which blocks early activation of calpain; only after the initiation of apoptotic execution, calpastain is cleaved by the activated caspase-3 [Wang et al., 1998; Porn-Ares

et al., 1998], which leads to activation of calpain and consequent cleavage of Bax. We are currently investigating this possibility by using both HL-60 and Jurkat cells. It should also be noted that cleavage of Bax into a p18 fragment was also observed in other apoptosis processes induced by irradiation or alphaviruses [Kirsch et al., 1999; Grandgirard et al., 1998].

We found that transfection of Bax/p21 cDNA did not induce apoptosis in either Jurkat T or MCF-7 cells (Fig. 5), which was different from some previous reports [Pastorino et al., 1998; Pastorino et al., 1999]. There are several possible interpretations for this result. First, it is possible that only a small portion of the overexpressed Bax/p21 was translocated to mitochondria under our conditions because we found only a small portion of the endogenous Bax/p21 in the mitochondrial membrane-bound fraction (Fig. 3D). The full-length Bax/p21 was also reported to be localized mainly in the cytosol under nonapoptotic conditions [Gross et al., 1999]. Second, it is possible that the small amount of the mitochondrial Bax/p21 is bound, and therefore inhibited, by endogenous Bcl-2 protein. Consistent with this argument, we found that the endogenous Bax/p21 was associated with Bcl-2 in the mitochondrial fraction (Fig. 3G). Third, it is possible that levels of endogenous Bcl-2 or Bcl-X_L proteins in our Jurkat T and MCF-7 cells are very high, which could inhibit the effect of the overexpressed Bax/p21. Finally, the level of the overexpressed Bax/p21 protein under our experimental conditions might still be below a threshold above which Bax/p21 overexpression could induce apoptosis. In any case, our data suggest that the Bax/p18 fragment is a more potent apoptosis inducer than the full-length Bax protein.

In addition to the described positive regulation of Bax by calpain during apoptosis in this study, most recently we have reported that Bax can also be negatively regulated in growing human cancer cells via the ubiquitin/proteasome-mediated degradation pathway, which is associated with tumor cell survival and progression [Li and Dou, 2000]. Our future studies will focus on how calpain-mediated Bax cleavage activity is activated by different apoptosis triggers, how Bax/p18 mediates release of mitochondrial cytochrome c to the cytosol, and whether calpain-mediated Bax cleavage under apoptotic conditions is related to proteasome-mediated Bax degradation under nonapoptotic conditions. In summary, because the mitochondria are the principal site of action for the Bcl-2 family proteins, the calpain-mediated Bax cleavage may be an essential step in mitochondria-dependent apoptosis.

ACKNOWLEDGMENTS

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