A growing body of evidence supports a role for mitochondria and mitochondria-derived factors in the cell death process. In particular, much attention has focused on cytochrome c, a key component of the electron transport chain, that has been reported to translocate from the mitochondria to the cytosol in cells undergoing apoptosis. The mechanism for this release is, as yet, unknown. Here we report that ectopic expression of Bax induces apoptosis with an early release of cytochrome c preceding many apoptosis-associated morphological alterations as well as caspase activation and subsequent substrate proteolysis. A loss of mitochondrial transmembrane potential was detected in vitro, although no mitochondrial swelling or loss of transmembrane potential was observed in isolated mitochondria treated with Bax in vitro. Caspase inhibitors, such as endogenous XIAP and synthetic peptide benzylxoycarbonyl-Val-Ala-Aspfluoromethyl ketone (zVAD-fmk), although capable of altering the kinetics and perhaps mode of cell death, had no influence on this release, suggesting that if cytochrome c plays a role in caspase activation it must precede this step in the apoptotic process. Mitochondrial permeability transition was also shown to be significantly prevented by caspase inhibition, indicating that the translocation of cytochrome c from mitochondria to cytosol is not a consequence of events requiring mitochondrial membrane depolarization. In contrast, Bcl-xL was capable of preventing cytochrome c release while also significantly inhibiting cell death. It would therefore appear that the mitochondrial release of factors such as cytochrome c represents a critical step in committing a cell to death, and this release is independent of permeability transition and caspase activation but is inhibited by Bcl-xL.

The stereotypical death throes of a cell undergoing apoptosis include DNA fragmentation, nuclear condensation, cell shrinkage, blebbing, and phosphatidylserine externalization (1–3), all features that promote the physiologically silent removal of the cell by its phagocytic neighbors. A large body of evidence supports the idea that these events are mediated by the activation of several cytosolic proteases, the caspases, which then orchestrate apoptosis via the cleavage of key substrates (reviewed in Refs. 4–7). For example, specific cleavage of two such substrates, PAR2 and DNA fragmentation factor, activate these proteins, mediating membrane blebbing and DNA fragmentation, respectively, without further requirements for the proteases (for these events) (8, 9).

But how are the caspases activated during apoptosis? Recent studies have delineated one key mechanism responsible for initiating the executioner phase of apoptosis. Early in the process, mitochondrial release cytochrome c (10), which upon entry into the cytosol forms a complex with another molecule, Apaf-1 (11, 12), and DNA fragmentation factor, activates this complex, PAK2 and DNA fragmentation factor, activate these caspases, which in turn can now trigger a cascade by processing and activating other caspases (in particular, caspases-3, -6, and -7) (13, 14). These then cleave key substrates and coordinate the process of apoptotic cell death.

Bax is a pro-apoptotic Bcl-2-family protein (15, 16) that resides in the cytosol and translocates to mitochondria upon induction of apoptosis (17, 18). Recently, Bax has been shown to induce cytochrome c release and caspase activation in vivo (19) and in vitro (20). This release was reportedly dependent upon induction of the mitochondrial permeability transition, an event associated with disruption of the mitochondrial inner transmembrane potential (ΔΨm) (21) and implicated in a variety of apoptotic phenomena (22–25). Bcl-2 was found to be capable of inhibiting Bax-induced apoptosis but not Bax-induced cytochrome c release in cells (19).

In contrast, Bax has been shown to be capable of blocking spontaneous cytochrome c release in cell-free extracts and in cells treated with apoptosis-inducing agents (26, 27). In the former, cytochrome c was able to completely bypass the anti-apoptotic effects of Bcl-2 (27). Furthermore, in both cell-free systems and in cells undergoing apoptosis, the release of cytochrome c can occur independently of changes in ΔΨm. We therefore examined the ability of Bax to induce the release of cytochrome c and apoptosis and evaluated the relationships between caspase activation, ΔΨm, and the effects of anti-apoptotic Bcl-2-family proteins.

MATERIALS AND METHODS

Cell Culture and Reagents—Human embryonic kidney cells (293T cells) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM l-glutamine under standard conditions. CEM cells were grown in RPMI medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Plasmid constructs pCDNA3, pCDNA3.Bax, and pCDNA3-myc-XIAP were generously provided by Dr. John Reed. Green fluorescent protein was purchased from CLONTECH (Palo Alto, CA).
Assessment of Mitochondrial \( \Delta \Psi \mathrm{m} \)—Changes in the inner mitochondrial transmembrane potential (\( \Delta \Psi \mathrm{m} \)) were determined by incubating \( 1 \times 10^5 \) cells in 40 nM of DiOC6 (3) or 150 nM MitoTracker Orange for 20 min at 37 °C. These two fluorochromes incorporate into cells dependent upon their mitochondrial transmembrane potential (29). The cells were then washed with PBS and excited using FACSscan flow cytometry. Controls were performed in the presence of 50 μM mitochondrial uncoupling agent mCClPP. In all cases, cells were gated to exclude cellular debris associated with necrosis. Assessment of mitochondrial transmembrane potential in isolated mitochondria was carried out by incubating 0.2 μg of mitochondria (prepared as described above) in 80 nM Rh123 for 20 min, scoring immediately by FACScan flow cytometry. Controls were performed in the presence of FCCP (1 μM).

Purification of Bax Protein—DH-5α bacterial cells containing a pGEX-KG expression vector with the murine Bax protein lacking the C-terminal hydrophobic region (BaxC19, amino acids 1–173) were treated with 0.1 mM isopropyl-1-thio-b-galactoside for 4–6 h at 37 °C. Bacterial cell pellets were lysed in 0.5 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 0.1 mg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin in PBS and sonicated for 4 min on ice (output 65.5; duty 90%). Cell lysates were then centrifuged at 20 000 × g for 20 min at 4 °C. The supernatant was loaded onto a glutathione-Sepharose-4B column and the column washed with PBS. Bound GST-Bax protein was eluted from the column with 80 μg/ml N\(_7\)-p-tosyl-L-lysine chloromethyl ketone protease inhibitor and dialyzed overnight against 20 mM Hepes-KOH pH 7.4, 10 mM KCl, 1.5 MgCl\(_2\), 1 mM dithiothreitol, 5 mM EDTA.

Cell-free Apoptosis with Isolated Mouse Liver Mitochondria—Mitochondria were isolated from liver tissue of 6-week-old Balb/c mice. Briefly, the livers were taken and homogenized with a Teflon glass potter in Buffer A (0.2 M mannitol, 0.05 M sucrose, 1 mM EDTA, 10 mM KCl, 5 mM succinate, 10 mM Hepes-KOH, pH 7.4, and 0.1% bovine serum albumin). All steps were then carried out at 4 °C. Samples were centrifuged at 1,030 × g for 15 min. The supernatant was transferred to another tube and centrifuged at 3,300 × g for an additional 10 min. Pellets were resuspended in Buffer B (0.3 M mannitol, 5 mM potassium phosphate, 10 mM Hepes-KOH, pH 7.4, and 0.1% bovine serum albumin) and centrifuged at 1,030 × g for 10 min. The supernatant was collected and centrifuged at 3,300 × g for 10 min. Finally mitochondrial pellets were resuspended in MSH buffer containing an ATP regenerating system (210 mM mannitol, 70 mM sucrose, 10 mM Hepes-KOH, pH 7.4, 0.2 mM EGTA, 5 mM succinate, pH 7.0, 0.15% bovine serum albumin, 2 mM ATP, 1 mM DATP, 10 μM phosphocreatine, 50 μM/μg creatine kinase, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The freshly isolated mitochondria were then incubated with recombinant Bax protein in the presence or absence of S-100 cytosolic extract. After 5 to 60 min at 37 °C, mitochondria were removed by centrifugation at 20 000 × g, and supernatants were analyzed by immunoblotting as described above.

Preparation of S-100 Extracts—Jurkat cells were grown for 3 days. Cell pellets were then resuspended in 20 mM extraction buffer (HEPES-KOH, pH 7.4, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM sodium-EDTA, 1 mM sodium-EGTA, 1 mM dithiothreitol, 250 mM sucrose, 10 mM succinate, 10 μg/ml leupeptin, 10 μg/ml aprotinin), incubated for 30 min on ice, and lysed by homogenization using a glass dounce (40 strokes/B-pestle). Cell debris were removed by centrifugation at 20,000 × g in an Eppendorf centrifuge for 15 min at 4 °C. Supernatants were re-centrifuged at 100 000 × g for 1 h in an Ultracentrifuge, and the resulting S-100 extract was stored at −70 °C.

Mitochondrial Swelling Assay—100 μg of freshly isolated mitochondria protein was incubated with various amounts of Bax protein in 500 μl of MSH buffer containing an ATP regenerating system, and \( A_{520} \) was measured over time (20, 30). A decrease in light scattering is consistent with an increase in mitochondrial volume. As controls for mitochondrial swelling, atracyloside (5 mM in MeSO) and CoCl\(_2\) (100 μM) were used.

**RESULTS**

Bax promotes apoptosis induced by removal of growth factors and other stimuli (15, 18, 31–33), and in some cases, ectopic expression can itself induce apoptosis (34–36). To confirm this, we transfected 293T cells with a construct for expression of Bax together with one for expression of green fluorescent protein (37). We monitored caspase activation in individual cells using a cell-permeable, fluorescent substrate (as described under “Materials and Methods”). As shown in Fig. 1A, Bax-transfected cells exhibited both morphological and biochemical char-

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1. The abbreviations used are: DiOC\(_6\), dihexyloxacarbocyanine iodide; mCICCP, carbamoyl cyanide n-chlorophenylhydrazone; DAPI, 4′,6-diamino-2-phenylindole dihydrochloride; FCCCP, carbamoyl cyanide p-(trifluoro-methoxy)phenylhydrazone; zVAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; PBS, phosphate-buffered saline; Pip, 1,4-piperazinediethanesulfonic acid.
characteristics of apoptosis. Cells induced to die upon Bax expression appeared rounded and blebbed while displaying condensed chromatin, fragmented nuclei, and active DEVD-cleaving caspases. We then examined whether expression of Bax could induce the release of mitochondrial cytochrome c. Fig. 1B shows that as the levels of Bax protein increased, cytochrome c could be detected in the cytosol. In other experiments including earlier time points before 9 h (not shown), negligible Bax expression was observed with neither cytochrome c nor caspase activation detectable.

We previously observed that the release of cytochrome c induced by staurosporine or UVB irradiation occurs before and independently of caspase activation and subsequent apoptosis (38). To examine this with respect to Bax-induced cytochrome c release, we treated Bax-transfected cells with the pan-caspase inhibitor zVAD-fmk. As described by others (39–43), zVAD-fmk significantly inhibited apoptosis during the period studied (24 h) (Fig. 2A). As shown in Fig. 2B, this was not because of any effect on Bax expression levels. Bax-induced apoptosis corresponded to the activation of caspases and the subsequent cleavage of fodrin and poly(ADP-ribose) polymerase, two caspase substrates previously shown to be cleaved during apoptosis (44), whereas the caspase inhibitor zVAD-fmk efficiently blocked this. Nevertheless, Bax-induced cytochrome c release proceeded with the same kinetics with or without caspase inhibition (Fig. 2B) as measured by cell fractionation and immunoblot analysis. This was confirmed by densitometric analysis of the cytochrome c immunoblots. At all time points, the cytosolic cytochrome c in the presence of Bax plus zVAD-fmk was ≥ that of Bax alone (data not shown).

As another approach to inhibiting caspase activation, we co-transfected Bax and a construct for expression of XIAP. Recent studies have shown that this molecule is a potent inhibitor of caspase function, including caspase activation by cytochrome c (45). As shown in Fig. 2, XIAP coexpression completely blocked apoptotic cell death (Fig. 2C) and caspase substrate cleavage (Fig. 2D) triggered by Bax. Nevertheless, XIAP expression had no effect on Bax-induced cytochrome c release (Fig. 2D). Once again zVAD-fmk was demonstrated to be capable of blocking caspase activation but had no effect on cytochrome c release.

Cell-free systems have been extremely valuable for the analysis of apoptosis mechanisms, including cytochrome c release from mitochondria (9, 10, 13, 26, 27, 46, 47). We therefore asked whether recombinant Bax protein can induce cytochrome c release in vitro and whether this is a direct or indirect effect of the protein. As shown in Fig. 3, the addition of Bax to cytosolic extracts containing mitochondria induced a rapid release of cytochrome c. Similarly, the addition of Bax to isolated mitochondria rapidly induced cytochrome c release, which was even more pronounced than that seen in the presence of cytosol. It appears, therefore, that this release is a direct effect of Bax on the mitochondria, as observed by others (19, 20). Interestingly, the presence of cytosol appeared to delay the Bax-induced release of cytochrome c from mitochondria in vitro. It is possible that this can be simply explained by levels of inhibitors in the cytosol, such as Bcl-2 or other Bax-binding proteins, which may sequester Bax and thereby interfere with its activity.

In many systems, apoptosis is associated with a loss of mitochondrial inner membrane potential (Δψm), which may correspond to the opening of an outer membrane pore (permeability transition pore). It has been suggested that this event is responsible for cytochrome c release (48), although we and others (26, 27, 38) have shown that such release can occur in the absence of a decrease in Δψm. Furthermore, we have shown that inhibition of caspase activation by zVAD-fmk can block early changes in Δψm without affecting cytochrome c release. In contrast, Xiang et al. (35) observed that the decrease in Δψm induced by Bax is unaffected by caspase inhibition with zVAD-fmk.

Therefore, we examined Δψm in our system using two fluo-
rochromes, DiOC$_6$(3) and MitoTracker Orange. As shown in Fig. 4A, Bax expression induced a loss of DC$_{mi}$, regardless of the fluorochrome employed. The addition of zVAD-fmk substantially decreased the number of cells displaying this loss, although some cells continued to show a loss of DC$_{mi}$ in the presence of zVAD-fmk, consistent with the observations of Xiang et al. (35). Similar results were obtained when caspases were inhibited by co-transfection with XIAP (Fig. 4C). Despite the persistence of this phenotype in some cells, these data suggest that the loss of DC$_{mi}$ is not required for Bax-induced cytochrome c release, because no change in this release was observed upon caspase inhibition (Fig. 2). The mitochondrial uncoupler, mCICCP, was used as a positive control for DC$_{mi}$ disruption (Fig. 4, B and D). To confirm this observation, we examined changes in ΔΨm using Rh123 and cytochrome c release by immunoblot after 18 h of Bax expression. 5 μg of isolated mitochondria fraction (Mito. Fr.) was used as a positive control for cytochrome c staining. Actin was used as loading control.

Recently Vander Heiden et al. (49) provided evidence that during apoptosis there is a disruption of the mitochondrial outer membrane, which may be responsible for the release of cytochrome c. One way this could happen would be through induction of swelling of the outer membrane until it breaks the outer membrane, as was suggested (49). We examined mitochondrial swelling as a function of light scattering, as described (20, 30). Although both CaCl$_2$ and atractyloside induced rapid swelling, we were unable to detect Bax-induced swelling of mitochondria in vitro (Fig. 5C), suggesting either that other mechanisms are involved or that any swelling is too transient to be detected by this technique.

Altogether this suggests that Bax can induce alterations in ΔΨm in vivo, but it does so indirectly and requires cytosolic participation. Caspases are proposed as one likely candidate for this phenomenon. One of the best indicators of the mitochondrial permeability transition is swelling of the organelle, which can be readily observed in isolated mitochondria. Our results (discussed above), which failed to show such swelling, provide further evidence against an irreversible permeability transition induced by Bax in vitro.

Recently two groups reported that Bcl-2 proteins can block apoptosis by acting downstream of cytochrome c (19, 50). In
light of this we therefore examined whether Bcl-xL, a member of the Bcl-2 family, could inhibit Bax-induced death and cytochrome c release. Bcl-xL inhibited cell death (Fig. 6A) when co-transfected with Bax, and this was accompanied by a prevention or delay in cytochrome c release (Fig. 6B). Caspase activation as determined by poly(ADP-ribose) polymerase cleavage was also inhibited by Bcl-xL.

The ability of Bcl-2-family members to interfere with Bax-
induced apoptosis was also observed *in vitro*. Mitochondria were prepared from CEM cells or CEM with ectopic expression of Bcl-2. Although Bax readily induced cytochrome c release from mitochondria isolated from the parental line, the mitochondria isolated from the Bcl-2-expressing cells were relatively resistant to this effect of Bax (Fig. 6).

Therefore, the induction of cytochrome c release from mitochondria by Bax is inhibited by Bcl-2 and Bcl-xL.

**DISCUSSION**

In this paper we have shown that ectopic expression of Bax induces mitochondria to release cytochrome c, caspase activation, and apoptosis. Although apoptosis depends upon caspase function, cytochrome c release does not, suggesting that if cytochrome c plays a role in caspase activation, it must precede this step in the apoptotic process. Several studies have shown that cytochrome c can trigger caspase activation in cell-free extracts (47), supporting this possibility.

In some systems, signals leading to apoptosis result in the transcription of key genes, which in turn lead to the death of the cell. Bax can function as one such gene. For example, DNA damage in some cells induces p53, which can activate the Bax promoter such that DNA damage results in elevation of Bax levels (51–54). Expression of Bax might then promote apoptosis via targeting mitochondria and inducing cytochrome c release.

In many cases, however, transcription is not required for apoptosis (55, 56). Nevertheless, Bax may play a crucial role in the apoptotic process via a number of different mechanisms. For example, Bcl-xL counteracts the effects of Bax (18, 57), and this molecule can be sequestered in the cytosol by another protein, BAD (58). Phosphorylation of BAD via growth factor receptor signaling and the Akt kinase releases Bcl-xL to target mitochondria (59). Thus, upon growth factor withdrawal, Bcl-xL becomes sequestered, and Bax may then be free to induce cytochrome c release and apoptosis. Not surprisingly, then, elevated levels of Bax exacerbate the effects of growth factor deprivation in cells (15, 31).

Some studies have shown that Bax translocates from its predominantly cytoplasmic location to the mitochondria (18) upon apoptosis induction. In cells overexpressing Bax, we similarly observed that this molecule remained mostly cytoplasmic for several h and then localized to the mitochondria around the time of cytochrome c release (approximately 12 h, data not shown). The signals and mechanisms responsible for this change in Bax distribution are not known, although our results would suggest that they can be caspase-independent, because caspase inhibitors did not block Bax-induced cytochrome c release (Fig. 2). Because Bax can clearly promote apoptosis, the nature of the translocation signal leading to induction of mitochondrial release of cytochrome c potentially takes on significance as a major apoptotic signaling pathway.

Once localized to the mitochondria, how does Bax induce the release of cytochrome c? Our studies support the idea that this can occur independently of a decrease in ΔΨm. Although Bax induced a dramatic decrease in mitochondrial transmembrane potential, inhibition of caspases by XIAP or zVAD-fmk significantly reduced the number of cells displaying such a loss while...
not affecting the extent of cytochrome c release. It is possible, however, that loss of ΔΨm occurs in two stages, a minor caspase-independent loss followed by a more dramatic caspase-dependent loss. Although this remains a possibility, it is noteworthy that we failed to detect any decrease in mitochondrial transmembrane potential in isolated mitochondria treated with Bax, despite the release of cytochrome c (Fig. 5).

Recent studies by Vander Heiden et al. (49) have suggested that during apoptosis, a hyperpolarization of the mitochondrial inner membrane causes a swelling that might act to puncture the outer membrane without necessarily disrupting ΔΨm in the short term. In this model, Bcl-xL acts as an ion channel (60, 61) to offset this hyperpolarization by allowing an efflux of protons. Bax can also act as an ion channel (62, 63), and thus, our results are consistent with the possibility that Bax promotes inner membrane swelling and outer membrane puncture. However, we were unable to detect the expected mitochondrial swelling (Fig. 4B). Either this is a transient effect that we were simply unable to capture or else Bax promotes a loss of mitochondrial outer membrane integrity via a different mechanism. A similar failure to detect Bax-induced mitochondrial swelling in vitro was recently described by Jürgensmeier et al. (20).

Studies by Xiang et al. (35) have shown that inducible Bax expression triggers a rapid caspase-dependent apoptosis, but if caspase activity is inhibited, a slower nonapoptotic death proceeds that is associated with generation of reactive oxygen species (whether or not these are responsible for the subsequent death still remains unknown). Bax-induced cytochrome c release helps to explain these observations. First, cytochrome c can trigger caspase activation and apoptosis (10, 13, 14, 47). In addition, however, and independently of caspase activation, the release of cytochrome c might be expected to result in disruption of electron transport, as has been observed in Fas-induced apoptosis. The resulting loss of ATP and generation of reactive oxygen species may ultimately cause cell death even in the absence of caspase function. This, of course, does not exclude the possibility that Bax has additional death-promoting activities. Nevertheless, the ability of Bax to induce the disruption of the mitochondrial outer membrane and the release of cytochrome c represents an important step in Bax-induced cell death.

Controversy over the mechanism of action of Bcl-2 and its homologue Bcl-xL has arisen recently with reports that these two anti-apoptotic oncogenes can block apoptosis by acting downstream of cytochrome c in the cell death pathway (19, 50). Bcl-2 has long been known to block cell death. Despite the vast literature dealing with this family of oncogenes, little is known about how the mechanisms used by these molecules prevent apoptosis. Some clues were provided by the observations that the structure of Bcl-xL resembles diphtheria toxin, which is able to form channels in cellular membranes (60). Furthermore, it was shown that Bcl-xL and Bcl-2 can indeed form ion channels in vitro (61). Previously it had been demonstrated that high levels of Bcl-2 can prevent the release of cytochrome c and, thus, caspase activation in response to a number of apoptosis-inducing stimuli, such as UVB, staurosporine, and etoposide (26, 27). Furthermore, in vitro, exogenous cyto-
chrome c bypassed this inhibitory effect of Bcl-2 (27). Similarly, Duckett et al. (64) showed that redistribution of cytochrome c is an early event in apoptosis that is inhibitable by Bcl-xL, but microinjection of cytochrome c overcomes this apoptotic inhibition. Together these studies proposed that a possible anti-apoptotic mechanism of Bcl-2 and its anti-apoptotic members was to inhibit cytochrome c translocation from mitochondria to the cytosol, thereby preventing caspase activation and subsequent apoptosis.

However Bcl-2 is not restricted exclusively to the mitochondrial membrane (65), and therefore, the possibility that this protein may have multiple anti-apoptotic mechanisms must be considered. To complicate matters, the Bcl-2 family members have been shown to bind to several proteins (66), not including other members of the Bcl-2 family, which may determine their localization and, therefore, activity. Reports that Bcl-xL can itself bind cytochrome c (67) and Apaf-1 (68) may possibly explain how apoptosis induced by cytochrome c microinjection could be inhibited in cells overexpressing Bcl-2. It remains possible that cells with high levels of Bcl-2 may have a large cytoplasmic fraction that is available to bind and thereby sequester exogenously added cytochrome c and, in doing so, quench its pro-apoptotic activity.

**FIG. 5.** Bax induces release of cytochrome c directly from mitochondria independently of mitochondrial depolarization and without mitochondrial swelling. A, time course analysis of mitochondrial permeability transition by Rh123 after freshly isolated liver mitochondria from Balb/c mice were incubated with recombinant Bax protein (30 μg/ml) in MSH for indicated periods of time. Each histogram represents the analysis of 50,000 events. The uncoupler, FCCP, was used as a positive control. B, immunoblot of cytochrome c release at the same time as mitochondrial membrane potential was examined. C, freshly isolated mitochondria were incubated with various amounts of Bax protein (■, 5 μg/ml Bax; ●, 10 μg/ml Bax; ▲, 20 μg/ml Bax; ●, 30 μg/ml Bax; ■, 40 μg/ml Bax), and A_{220} was measured over time. As controls for mitochondrial swelling, atractyloside (Atr) 5 mM in Me_{2}SO and CaCl_{2} (C, 100 μM) were used. A decrease in OD_{220} is consistent with an increase in mitochondrial volume.

**FIG. 6.** Bcl-2 family members protect cells from Bax-induced death and release of cytochrome c. A, 293T cells were co-transfected with 1 μg of Bax and 2 μg of Bcl-xL and analyzed for DNA fragmentation at specific time points post-transfection. ○, vector; ■, Bcl-xL; ●, Bax; ■, Bax + Bcl-xL. B, Western blot analysis of cytochrome c release and poly(ADP-ribose) polymerase (PARP) cleavage after Bax expression in the presence or absence of Bcl-xL. Actin was used as loading control. C, 5 μg of mitochondria isolated from CEM.neo or CEM.Bcl-2 cells were incubated with recombinant Bax protein (30 μg/ml) in MSH for indicated periods of time. 5 μg of isolated mitochondria (Mito. Fr.) was used as a positive control for cytochrome c staining.
In contrast, we observed that Bcl-xL can significantly delay the release of cytochrome c from mitochondria (and subsequent apoptosis) in response to ectopic Bax. These results parallel similar observations reported in yeast studies (57). The anti-apoptotic mechanism, however, has not yet been determined. Proposed mechanisms include the formation of heterodimers between Bcl-2/Bcl-xL and Bax (15), which would interfere with the availability and translocation of the Bax protein from the cytoplasm to the mitochondria. Alternatively, counteracting the availability and translocation of the Bax protein from the mitochondrial membrane, however, has not yet been determined.

In conclusion, we have shown that Bax induces the release of cytochrome c in conjunction with apoptosis and that caspase inhibition, although altering the kinetics and perhaps mode of cell death, has no effect on this release. In contrast, members of the anti-apoptotic oncogene family, Bcl-2 and Bcl-xL, are capable of inhibiting or delaying this release while significantly preventing cell death. This suggests that Bcl-2 family members may play a modulating role in blocking the mammalian cell death machinery by acting upstream of caspase function and upstream at the mitochondrial level.

Acknowledgments—We thank Drs. A. Gross and S. Korsmeyer for their pGEX-KG-BaxC19 construct and advice in the isolating Bax protein. We also thank Dr. Ruth Kluck for helpful discussions.

REFERENCES

1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
2. Wyllie, A. H. (1980) Br. J. Exp. Pathol. 61, 335–341
3. Martin, S. J., and Green, D. R. (1995) J. Biol. Chem. 270, 11357–11362
4. Martin, S. J., Lennon, S. N., Williams, E. A., and Henson, M. P. (1996) J. Biol. Chem. 271, 11131–11138
5. Martin, S. J., and Korsmeyer, S. J. (1997) Science 278, 796–802
6. Martin, S. J., and Korsmeyer, S. J. (1998) Science 281, 1318–1320
7. Martin, S. J., and Korsmeyer, S. J. (1999) Cell 96, 513–516
8. Martin, S. J., and Korsmeyer, S. J. (2000) Science 289, 1318–1320
9. Martin, S. J., and Korsmeyer, S. J. (2001) J. Exp. Med. 193, 655–662
10. Martin, S. J., and Korsmeyer, S. J. (2002) Apoptosis 7, 7–22
11. Martin, S. J., and Korsmeyer, S. J. (2003) Cell 116, 959–973
12. Martin, S. J., and Korsmeyer, S. J. (2004) Nature 421, 757–763
13. Martin, S. J., and Korsmeyer, S. J. (2005) Cell 122, 707–709
14. Martin, S. J., and Korsmeyer, S. J. (2007) Science 315, 365–369
15. Martin, S. J., and Korsmeyer, S. J. (2008) Nature 453, 1137–1139
16. Martin, S. J., and Korsmeyer, S. J. (2009) Cancer Res. 69, 108–110

Bcl-xL Inhibits Bax-induced Cytochrome c Release

Bcl-xL can significantly delay the release of cytochrome c from mitochondria in response to ectopic Bax. This result parallels similar observations reported in yeast studies. Proposed mechanisms include the formation of heterodimers between Bcl-2/Bcl-xL and Bax, which would interfere with the availability and translocation of the Bax protein from the cytoplasm to the mitochondria. Alternatively, counteracting the availability and translocation of the Bax protein.
