We have shown previously that Bcl-XS causes acute cell death in 3T3 cells without activating caspases (Fridman, J. S., Benedict, M. A., and Maybaum, J. (1999) Cancer Res. 59, 5999–6004). In this study, we determined that the explanation for lack of caspase activation is the cellular depletion of cytochrome c. Electron microscopy revealed gross structural changes in the mitochondria of Bcl-XS-expressing cells; however, cytochrome c was not detected in cytosolic fractions from these cells. Surprisingly, it was determined that cellular cytochrome c levels decreased as Bcl-XS expression levels increased. Experiments performed to eliminate other possible explanations for the lack of caspase activation showed that these 3T3 cells have a functional cytosolic apoptosome, a complex of proteins that form a functional trigger capable of activating the proximal caspase in an apoptotic pathway Chinnaiyan, A. M. (1999) Neoplasia 1, 5-15, as cytosolic extracts from these cells were capable of cleaving pro-caspase-9. These cells were also able to release cytochrome c from their mitochondria after appropriate stimulation, other than Bcl-XS expression (i.e. withdrawal from serum for 24 h), and initiate a cell death that is inhibited by a dominant negative caspase-9. We conclude that lack of caspase activation is due to a Bcl-XS-induced depletion of active cytochrome c, a phenomenon that represents an alternative cell death effector pathway and/or a novel mechanism for regulating caspase activation.

Bcl-XS, belongs to the Bcl-2 family of proteins, whose members can either promote or inhibit cell death, depending on their structural features. These features are referred to as Bcl-2 homology (BH) domains and are highly conserved throughout this family of proteins. The pro-survival proteins, Bcl-2 and Bcl-XL, contain a BH1, BH2, BH3, and a BH4 domain, whereas the pro-death members of the family have, at least, a BH3 domain. The BH1, BH2, and BH3 domains form a hydrophobic binding pocket into which a BH3 domain of another family member binds, forming either a hetero- or a homodimer (3, 4). It has been proposed that the ratio of the pro-survival to pro-death members of this family determines the propensity of a cell to live or die (5, 6), although the specific mechanisms by which a pro-death or a pro-survival family member act are still poorly understood.

The most extensively studied pro-death family member, Bax, has a BH1 and a BH2 domain in addition to the obligatory BH3 domain. These domains (BH1 and BH2) are part of the proposed membrane-spanning domain that forms channels in synthetic membranes and lipid vesicles (7, 10). The ability to form such channels is one of the proposed models by which Bax may act (8, 9, 11–13).

Bcl-XS, in contrast to Bax, lacks BH1 and BH2 domains. Therefore, Bcl-XS should not form membrane-spanning channels and thus acts through a different mechanism. Bcl-XS does, however, have a BH3 domain as well as a BH4 domain (thought to allow for protein-protein interactions outside the Bcl-2 family), a unique combination among the Bcl-2 family members. Although there are no other known pro-death Bcl-2 family members that have only BH3 and BH4 domains, there are a number of BH-3-only proteins including, Hrk, Bik, Bad, and EGL-1. These proteins are thought to act either by binding to and inactivating the pro-survival functions of Bcl-2 and Bcl-XL (or related proteins), or by displacing pro-death Bcl-2 family members that are capable of forming transmembrane channels, from Bcl-2 or Bcl-XL, thereby allowing them to kill the cell. If either of these models is correct, then the outcome will be disruption of the functionality and/or integrity of organelles in which these proteins are concentrated, the mitochondria and endoplasmic reticulum.

Mitochondria have been the focus of the majority of studies aimed at explaining the role of the Bcl-2 family in cell death. Evidence for such a role includes the following; 1) cell death has been correlated with loss of the mitochondrial membrane potential (ΔΨm), and 2) release of apoptogenic factors including cytochrome c, AIF, and caspases has been observed upon mitochondrial disruption. The mechanism by which Bcl-2-related proteins affect the mitochondria is not clear. However, it has been shown that Bax can cause release of cytochrome c from mitochondria, whereas Bcl-2 and Bcl-XL can prevent such a loss induced by Bax or other insults (in isolated mitochondria and in whole cells) (6, 13, 14). Cytochrome c, once released into the cytosol, becomes part of an apoptosome by binding Apaf-1 in an ATP-dependent manner. This binding allows for the oligomerization of Apaf-1 molecules and the recruitment of pro-caspase-9. Pro-caspase-9 is then thought to be activated by a proximity model that utilizes the low but significant catalytic activity of the pro-caspase (15, 16). This initiates a caspase cascade and apoptosis.

We have shown recently that Bcl-XS can kill 3T3 cells and that this cell death does not require, nor does it activate, caspases (1). In that report we proposed four possible mechanisms of action for Bcl-XS in 3T3 cells (see Fig. 1). The first of these models, the activation of caspases through the release of...
membrane-spanning channels.

domain (such as Bax), which might then act through one of the first two effector caspases; 2 release of cytochrome as a result of physical association; 2 h at room temperature or overnight at 4 °C. After each incubation, upon expression of Bcl-XS and to determine which, if any, of study, we sought to determine why caspases are not activated however, we did find that expression of Bcl-XS was temporally coincident with collapse of the mitochondrial membrane potential (ΔΨm) and was therefore affecting the mitochondria. In this study, we sought to determine why caspases are not activated upon expression of Bcl-XS and to determine which, if any, of the proposed mechanisms (Fig. 1) is responsible for Bcl-XS-mediated death in 3T3 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Tissue Culture**—3T3XS7.2 cells and stable derivatives of these cells expressing Bcl-XL or dominant negative caspase-9 (9DN) were derived and maintained as described previously (1). Electron Microscopy—Approximately 5 × 10^6 3T3XS7.2 cells, or their Bcl-XL-overexpressing derivatives, were plated in 100-mm tissue culture dishes and allowed to grow for 24–48 h in the presence of tetracycline (TET). In the TET withdrawal time-course experiment, plates were withdrawn from TET at different times so that all samples were collected at the same time.

After removal of medium, cell monolayers were fixed with 2% glutaraldehyde in 0.1 M Sorensen’s buffer, pH 7.4. The aspirated media was centrifuged to pellet floating cells that were subsequently pooled with their respective adherent cells. Cells were fixed in 1% osmium tetroxide and dehydrated in a graded series of ethanol. Cell pellets were embedded in Spurr’s resin. Ultrathin sections, showing a silver interference color, were collected and stained with uranyl acetate and lead citrate. Sections were viewed on a Philips CM100 electron microscope.

**Preparation of Cytosolic Extracts**—Cytosolic extracts were prepared by washing cells twice in cold phosphate-buffered saline. All additional steps were performed at 4 °C or on ice. Cells were resuspended in cold buffer A (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM Na-EGTA, 1 mM Na-EDTA, 1 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, 220 mM mannitol, 200 mM sucrose) and Complete protease inhibitors (Roche Molecular Biochemicals) and left for 40 min on ice. Cells were then disrupted by Dounce homogenization (45 strokes). The solution was then centrifuged twice at 500 × g to remove nuclei and unlysed cells. The supernatant was then spun at 14,000 rpm in a microcentrifuge at 4 °C for 30 min. The supernatant, referred to as the cytosolic extract, was removed and stored at −80 °C until used. The pellet was also saved and stored at −80 °C and is considered to contain the membrane fraction greatly enriched in mitochondria.

**Immunoblot Analyses**—Protein concentrations for cytosolic extracts were normalized and loaded onto SDS-polyacrylamide gels. For whole cell samples, cells were counted, centrifuged at 500 × g, and resuspended in sample buffer (17) and boiled. Equivalent numbers of cells were loaded onto SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (equal protein loading was confirmed by Ponceau-S staining) and blocked for at least 2 h in TBST (Tris-buffered saline and 0.1% Tween 20) containing 5% (w/v) milk. All primary antibodies were diluted in TBST containing 1% milk, and incubations were carried out for either 2 h at room temperature or overnight at 4 °C. After each incubation, membranes were washed six times in TBST. Anti-cytochrome c antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:500 or 1:1000 (PharMingen, San Diego, CA), anti-Hsp60 antibody (Stressgen Incorporated) was used at 1:2000, and anti-VDAC (porin) (Calbiochem) was used at 1:200. Secondary antibodies were used at 1:50,000 (Pierce). *Cytochrome c Depletion upon Expression of Bcl-XS.*

**Possible models of Bcl-XS-induced cell death.** 1, direct release of cytochrome c from mitochondria and subsequent activation of effector caspases; 2, inhibition of anti-apoptotic Bcl-2 family members as a result of physical association; 3, liberation/activation of one or more pro-apoptotic Bcl-2 family members which possess a BH1 and BH2 domain (such as Bax), which might then act through one of the first two models (or the last); or 4, formation of new (or alteration of existing) membrane-spanning channels.

**Quantification of Cellular ATP Levels by High Performance Liquid Chromatography (HPLC)**—Cells were grown in the presence or absence (−TET) of tetracycline for the indicated period of time. +Fas/Act D cells are 3T3XS7.2 cells treated with activating anti-Fas antibody (500 ng/ml) and actinomycin D (4 μM) for 8 h in the presence of TET. Medium was saved from the culture dishes to retain any floating cells. Adherent cells were harvested and combined with their respective floating cell populations. Nucleotides were extracted from an equal number of cells with ice-cold 4% perchloric acid and subsequently neutralized. Nucleotides were separated and quantified by strong anion-exchange HPLC using a Waters (Milford, MA) gradient system controlled by Millenium 2010 software (20). Cellular ATP levels were quantified by comparison of their peak areas with that of a known amount of standard.

**Electron Transfection Death Assay**—3T3XS7.2 or 9DN cells (stable derivatives of the 3T3XS7.2 cells expressing a dominant negative caspase-9) were plated in six-well dishes and allowed to grow for 24–48 h. Cells were maintained in the presence of TET (1 μg/ml tetracycline) and were transfected with 0.825 μg of 35S-caspase-9, in the presence or absence of bovine cytochrome c (Sigma) and dATP. Reaction tubes were incubated and at 30 °C for 60 min, and the reaction was stopped by addition of 15 μl of 5 × sample buffer and boiling. Samples were loaded onto a SDS-polyacrylamide gel or frozen at −80 °C. Electrophoresed gels were dried, fixed, and exposed to BioMax MS film using a Transcreen LE intensifying screen (Eastman Kodak Co.) at −80 °C for 2–8 days.

**RESULTS**

**Structural Changes to 3T3 Cells upon Expression of Bcl-XS**—We have shown previously that enforced expression of Bcl-XS causes a loss of mitochondrial membrane potential (ΔΨm) in 3T3 cells, and that this occurs without subsequent activation of caspases (1). To look more closely at the mitochondrial changes, electron microscopy was performed on 3T3XS7.2 cells (a stable derivative of 3T3 cells expressing Bcl-XS in a
Independent experiments.

Deposits, marked M, and those showing structural abnormalities are marked M*. Cells showing structural and functional alterations also formed lipid deposits, marked L. Cells overexpressing Bcl-XL do not show disruption of their mitochondrial structure. These results are representative of two independent experiments.

The morphology of Bcl-XS-expressing cells appeared less electron-dense or even transparent (Fig. 2, marked M*), indicating lack of protein and membrane content. The structure of the cristae, in the mitochondria in which cristae were still visible, no longer filled the interior of the mitochondria. The cristae appeared broken and/or pushed against the outer mitochondrial membrane. In contrast, the mitochondria of the control cells (+TET) (Fig. 2, marked M) appeared electron-dense and, therefore, contain a substantial amount of membranes and protein. The cristae were numerous, intact, and healthy, as they spanned all regions of the mitochondria. The effects of Bcl-XS expression on the mitochondria were negated by the stable expression of Bcl-XL in these cells (Fig. 2, bottom); however, they were not prevented by pretreatment with the broad-spectrum caspase inhibitor z-VAD-fmk (up to 100 μM) or by expression of a dominant negative caspase-9 (data not shown). Expression of β-galactosidase in the identical expression vector also did not affect the mitochondria (data not shown).

In cells expressing Bcl-XS we also observed large, opaque, vesicles that are not membrane-bound (Fig. 2, marked L). These masses are lipid deposits often seen during mitochondrial distress (21-24) and do not arise in control (+TET) cells or β-galactosidase-expressing cells (data not shown). Pretreatment with z-VAD-fmk or expression of dominant negative caspase-9 did not prevent the formation of these vesicles (data not shown); however, expression of Bcl-XL did preclude their appearance (Fig. 2). These Bcl-XL-expressing cells also did not show a loss of ΔΨm upon expression of Bcl-XS, as opposed to the parental 3T3XS7.2 cells.

Depletion of Cytochrome c upon Expression of Bcl-XS in 3T3 Cells—The morphologic changes in the mitochondria upon expression of Bcl-XS (and the depolarization of the mitochondria), led to the assumption that cytochrome c was being released into the cytosol of the cells. This should have initiated the formation and activation (in the presence of sufficient ATP) of the Apaf-1-cytochrome c-caspase-9 apoptosome (22, 23). The fact that 9DN could inhibit cell death induced by withdrawal from serum suggested that such an apoptosome was functional in these cells. Therefore, the previously observed lack of caspase activation upon Bcl-XS expression was unanticipated (1).

To determine whether cytochrome c was being released into the cytosol upon expression of Bcl-XS, cytosolic extracts were made from 3T3XS7.2 cells at various times after withdrawal from TET, or after withdrawal from serum for 24 h. Withdrawal from serum for 24 h caused approximately the same extent of cell death (as assessed morphologically and/or by trypan blue exclusion) as did withdrawal from TET for 36 h (data not shown).

Release of cytochrome c into the cytosol was not detected upon expression of Bcl-XS (Fig. 3A). In contrast, 24 h after serum withdrawal, cytochrome c was easily detected in the cytosol. These results explain why serum withdrawal-induced death, but not death induced by expression of Bcl-XS, is inhibited by 9DN (data not shown and Ref. 1). However, the observed loss of ΔΨm (1) and the apparent destruction of the mitochondrial structure (Fig. 2) did not correlate with lack of detectable cytochrome c release.

To investigate the possibility that cytochrome c was not being released from the mitochondria because there was no cytochrome c to be released, we made whole cell extracts from the same cells used to make cytosolic extracts above (Fig. 3B).

Upon expression of Bcl-XS, first detectable between 24 and 30 h after withdrawal from TET (Fig. 3C) (1), the cellular levels of cytochrome c decreased slightly. This decrease became more pronounced as the time of withdrawal from TET increased. Cytochrome c was barely detectable 36–48 h after withdrawal; however, it was still present after withdrawal from serum for 24 h. To circumvent the possibility of a lack of antibody reactivity due to cytochrome c modification(s), we used two antibodies for immunoblot analyses, a polyclonal antibody raised against the full-length protein and a monoclonal antibody raised against three unique regions of the protein. Two extraction buffers, a SDS-based buffer and a Nonidet P-40-based buffer, were used for cytochrome c immunoblots and gave identical results (data not shown). Additionally, we took advantage of the covalently bound iron atom in cytochrome c and its inherent peroxidase activity to oxidize a chemiluminescent substrate (ECL) (18, 19). Using this technique we show that a band that comigrates with (and is the expected size of) purified bovine cytochrome c is also depleted upon expression of Bcl-XS, with similar kinetics as in the Western blot (Fig. 3D).

This decrease in cytochrome c was not common to all mitochondrial proteins as the mitochondrial matrix protein Hsp60 and the outer membrane protein VDAC were not depleted after withdrawal from TET (Fig. 3E). Additionally, confocal microscopy showed that the mitochondrial intermembrane space protein AIF was redistributed in the cytoplasm, but was not degraded upon expression of Bcl-XS (data not shown). These experiments led to the hypothesis that Bcl-XS does not induce caspase activation because cytochrome c is not present to a sufficient degree in the cytosol of Bcl-XS-expressing cells to complete and activate the mitochondrial apoptosome(s).

Addition of Cytochrome c Rescues the Ability of Cytosolic Extracts to Cleave 35S-Pro-caspase-9—To confirm that the lack of detectable cytochrome c, in the cytosolic fractions from Bcl-XS-expressing cells, was the explanation for absence of caspase activation in these cells, in vitro pro-caspase-9 cleavage assays were performed using cytosolic extracts from 3T3XS7.2 cells...
for the presence of cytochrome c in the absence of fetal bovine serum, marked and bovine cytochrome presence, but not in the absence of exogenously added dATP TET for 36 h) to cleave 3T3XS7.2 cells expressing Bcl-XS. Immunoblots were performed on 3T3XS7.2 cells (A and B) grown in the presence or absence of TET for the indicated times, or grown in the absence of fetal bovine serum, marked S, as a control for caspase-9-mediated cell death. Cytosolic (A) or whole cell extracts (B) were assayed for the presence of cytochrome c using a polyclonal antibody against cytochrome c. Expression of HA-tagged Bcl-XS was examined at the indicated time after withdrawal from TET with an anti-HA antibody. The presence of cytochrome c in cytosolic extracts was also examined directly by utilizing the peroxidase activity of the heme group covalently bound to cytochrome c and chemiluminescence (D). Positive controls (A, B, and D) are purified bovine cytochrome c. Immunoblots of two other mitochondrial proteins, Hsp-60 and VDAC (E), demonstrate that depletion of cytochrome c is not a general effect of all mitochondrial proteins. These results are representative of at least two independent experiments.

Cytosolic extracts from either 293T cells or 3T3XS7.2 cells were able to process 35S-labeled human pro-caspase-9 in the presence, but not in the absence of exogenously added dATP and bovine cytochrome c (Fig. 4A). The presence of two cleavage products upon apoptosis activation is the result of both auto-processing (caspase-9 cleavage of pro-caspase-9) and processing by downstream effector caspases, such as caspase 3 (25). These results confirm that a cytosolic apoptosome is functional in these cells and that complementation of these extracts with cytochrome c and dATP is sufficient to activate the proximal caspase in a cell death pathway.

We also looked at the ability of cytosolic extracts from 3T3XS7.2 cells expressing Bcl-XS (−TET for 36 h) to cleave pro-caspase-9. These extracts were also able to cleave pro-caspase-9 in the presence, but not in the absence, of exogenously added cytochrome c and dATP (Fig. 4B). Finally, an alternative explanation for the lack of caspase activation in these cells (upon expression of Bcl-XS) could be the presence of some type of inhibitor in these cells. These experiments also serve to disprove this possibility as such an inhibitor (e.g., an inhibitor of apoptosis) should have also prevented the processing of pro-caspase-9 in vitro. We do, however, recognize the possibility that an inhibitor may exist and that it was lost during the preparation of the cytosolic fraction. However, the fact that these cells die upon withdrawal from serum and that this death is inhibited by 9DN argues that the apoptosome is functional in whole cells (1).

**ATP Levels in 3T3XS7.2 Cells upon Expression of Bcl-XS or Treatment with Fas/Act D**—To strengthen the hypothesis that lack of detectable caspase activity in the presence of Bcl-XS expression is due to lack of cytochrome c in the cytosol, we examined another possible explanation, insufficient ATP levels. ATP is required for the oligomerization of Apaf-1 and activation of pro-caspase 9 (22, 23, 25). We measured ATP levels in cells that were dying by induction of Bcl-XS expression (noncaspase mediated) or by a caspase-mediated cell death induced by treatment with Fas/Act D. Nucleotides were extracted from 3T3XS7.2 at various times after withdrawal from TET, or after 8 h of Fas/Act D treatment (in the presence of TET). Extracted nucleotides were resolved and quantified by strong anion-exchange HPLC. The results show no significant decrease in the levels of ATP from whole cells upon expression of Bcl-XS out to 48 h (Table I). There was, however, an approximate 50% decrease in the amount of ATP extracted from cells treated with Fas/Act D. These cells have previously been shown to have significant caspase activity at this time point (1). As caspase activity is an energy-consuming process, the decreased ATP levels upon Fas/Act D treatment are not a surprise. However, it is interesting that, after structural disruption of the mitochondria and loss of the \( \Delta \Psi_m \) Bcl-XS-expressing 3T3 cells do not show a decrease in ATP levels. In fact, there is a reproducible increase in the cellular ATP content. This increase may result from either an increase in the production of ATP or from a decrease in its utilization/degradation. Considering the ob-
served loss of ΔΨm and the perturbations in the mitochondria upon expression of Bcl-XS, it seems more likely that the cause is a decrease in the utilization of ATP.

**Bcl-XS and Bax Kill through Nonoverlapping Mechanisms**—After determining that Bcl-XS kills 3T3 cells without caspase activation, because of a depletion of cytochrome c, we wanted to address another of the mechanisms proposed for Bcl-XS-induced cell death (Fig. 1). One of the described mechanisms entails the displacement, by Bcl-XS, of a pro-death Bcl-2 family member that possesses potential membrane-spanning domains, such as Bax. To test if Bcl-XS kills 3T3 cells by displacing such a protein, we performed the following experiments. Stable cell lines expressing 9DN were derived from our 3T3XS7.2 cells (1). The 9DN cells were shown to express dominant negative caspase-9 both by immunoblot analysis and functionally, by resistance to serum withdrawal (1). The 3T3XS7.2 cells and the 9DN cells were transiently transfected (in the presence of TET) with Bcl-XL, Bcl-XL, or Bax, along with a plasmid coding for a green fluorescent protein (pK7GFP) at a ratio of 3:1. The presence of TET throughout this experiment ensures that the TET-dependent expression of Bcl-XS is turned off and only the transiently transfected transgenes are expressed (data not shown). Twenty-four hours after transfection, the cells were examined by fluorescent microscopy. Green cells were scored morphologically as either live (flat cells with normal stellate morphology) or dead (rounded or floating) (Fig. 5). Two hundred cells were counted wherever possible, but, in three of four experiments, 200 surviving green cells could not be found in the Bax transfected 3T3XS7.2 cells. This is in comparison to thousands of green cells in the other conditions, including the Bax transfections of the 9DN cells (data not shown).

Analysis of variance showed that day-to-day variation was marginally significant ($p = 0.064$), indicating the importance of comparing results obtained on the same day of experimentation. Therefore, all data are paired, allowing comparison of results from the individual experiments. 9DN cells were slightly more resistant than the 3T3XS7.2 cells to base-line transfection cytotoxicity with Bcl-XL. However, there was no difference between cell lines transfected with Bcl-XS. The hypothesis that 9DN protects against Bax-induced, but not Bcl-XS-induced, cell death was tested using a statistical interaction hypothesis that 9DN protects against Bax-induced, but not Bcl-XL-induced, cell death (since it is not inhibited by a dominant negative caspase 9) proceeds, at least in part, through a pathway that does not utilize Bax or another Bcl-2 family member which requires a functional caspase 9. These data argue against the proposed model for Bcl-XS-induced cell death in which Bcl-XS displaces another pro-death Bcl-2 family member (which then kills the cell), such as Bax.

**TABLE I**

| Treatment       | Cellular ATP content (relative to control) |
|-----------------|--------------------------------------------|
| Control         | 100                                        |
| -TET (24 h)     | 95.5 ± 3.2                                 |
| +TET (36 h)     | 134.3 ± 9.4                                |
| +TET (48 h)     | 131.5 ± 17.3                               |
| +Fus/Act D (8 h)| 51.5 ± 5.3                                 |

**FIG. 5. Bcl-XS does not kill by displacing Bax.** 3T3XS7.2 cells, or their 9DN-expressing stable derivatives, were transiently transfected with plasmids to express Bcl-XL, Bcl-XS, or Bax, and a GFP reporter construct at a ratio of 3:1. After 24 h, green cells were scored as either live or dead. Four independent experiments using two different 9DN clones are shown, with the 7.2 and 9DN samples for each experiment connected by a line.

**DISCUSSION**

This study presents two important findings regarding Bcl-XS-induced cell death and the regulation of cell death as a whole. The most significant finding is the cellular depletion of cytochrome c that is temporally coincident with detectable expression of Bcl-XS and that prevents caspase activation during Bcl-XS-induced cell death in 3T3 cells. Second, Bcl-XS was shown to kill 3T3 cells by a pathway distinct from Bax and, therefore, does not require the activity of a pore-forming Bcl-2 family member, such as Bax. These findings support a model for the mechanism of action of Bcl-XS in 3T3 cells that entails inhibition of the death-suppressing effects of Bcl-2 or related proteins (i.e. a dominant negative Bcl-2) by direct interaction with such proteins (27, 28).

The observed depletion of cytochrome c is a novel mechanism for the prevention of caspase activation. Although various structural alterations to cytochrome c have been observed in other systems during programmed cell death, such as Jurkat cells (29, 30) and in Drosophila (26), in neither system was cytochrome c depleted from the cells, nor was caspase activation prevented. In the present study, complementation of cytosolic extracts (from 3T3XS7.2 cells expressing Bcl-XS) with exogenous cytochrome c restored the ability of these extracts to process pro-caspase-9, confirming that the lack of cytosolic cytochrome c was the explanation for the lack of caspase activation.

Of the four models to explain how Bcl-XS can kill 3T3 cells presented in this study (Fig. 1), the data described previously (1) disproved the first hypothesis, the activation of caspases, whereas the data presented here describe the mechanism by which caspase activation is prevented. Evidence supporting this mechanism, the depletion of cytochrome c, includes the following: First, there is a temporal coincidence between expression of Bcl-XS and the depletion of cytochrome c from 3T3XS7.2 whole cell extracts. This was confirmed by immunoblot analysis (using both monoclonal and polyclonal antibodies against cytochrome c) and directly, utilizing the peroxidase activity of the covalently bound iron atom in the heme group of cytochrome c and a chemiluminescent substrate, which is activated upon oxidation. The loss of detectable cytochrome c also occurs as there are both structural and functional perturbations detected in the mitochondria. Second, complementation of cytosolic extracts, from 3T3XS7.2 cells, with exogenous cytochrome c triggers processing of pro-caspases. Third, a mechanism for the release of cytochrome c from the mitochondria and the subsequent activation of caspase-9 and cell death is functional in these cells, as they have been shown to release cytochrome c into the cytosol upon serum withdrawal. Serum with-
Cytochrome c Depletion upon Expression of Bcl-XS

drawal-induced death in these cells was prevented by expression of 9DN; however, 9DN had no effect on Bcl-XS-induced cell death. Finally, alternative mechanisms for the inhibition of caspase activation were addressed and eliminated. 3T3XS7.2 cells can die by caspase-dependent pathways, as activation of the Fas receptor, serum withdrawal, or transfection with Bax have all been shown to kill in a caspase-dependent manner. These cells have functional apoptosomes and, as we have also demonstrated here, their ATP levels are not depleted, even after prolonged expression of Bcl-XS. Therefore, 3T3XS7.2 cells expressing Bcl-XS have all of the cellular machinery and the fuel to execute the apoptotic pathway, except for cytochrome c.

Interestingly, the depletion of cytochrome c appears to be a relatively specific phenomenon as other mitochondrial proteins, each with different localization within the mitochondria, VDAC, Hsp60, and AIF, were not depleted upon expression of Bcl-XS. It is not unreasonable to envision a mechanism present in either the mitochondria or in the cytosol that rapidly degrades or grossly modifies cytochrome c released (or when release is anticipated) in an environment otherwise not conducive to triggering programmed cell death.

Finally, these studies were initiated with the goal of determining the mechanism of action of Bcl-XS-induced cell death. Thus far we have disproved the activation of caspases as a possible mechanism, and we have also described the novel finding of depletion of cytochrome c as a method of inhibiting caspase activation upon Bcl-XS expression. In this study we also addressed another of the proposed mechanisms for Bcl-XS-induced cell death (Fig. 1), that Bcl-XS acts by displacing a pro-death Bcl-2 protein, such as Bax, that contains membrane-spanning domains. To address this model we used the 3T3XS7.2 cells and stable derivatives of these same cells expressing 9DN to determine whether expression of 9DN had a protective effect upon transfection with Bcl-XS or Bax. If Bcl-XS kills by displacing Bax, the protective effects of 9DN observed for Bax-induced death would also be observed upon transfection with Bcl-XS; however, this was not the case. The lack of protection, by 9DN, from Bcl-XS implies that Bcl-XS does not kill indirectly by the liberation and/or activation of a Bax-like protein. These findings, combined with the fact that Bcl-XS does not possess membrane-spanning domains and, therefore, cannot form membrane-spanning channels, supports a model in which Bcl-XS acts as a dominant negative Bcl-2/Bcl-XL and inhibits the pro-survival function of these proteins.

In summary, these findings suggest a novel pathway for cell death induced by a Bcl-2 family member, Bcl-XS, that does not utilize caspases. Additionally, there is evidence to suggest the existence of a novel regulatory mechanism, the depletion of cytochrome c, for the activation of caspases. Such a mechanism may have evolved in eukaryotes to safeguard the cell from an untimely demise upon the accidental release of cytochrome c. It will be interesting to determine what activity is responsible for the degradation of cytochrome c in these cells and to determine whether evidence exists in vivo for such mechanisms of regulating cell death.

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REFERENCES

1. Fridman, J. S., Benedict, M. A., and Maybaum, J. (1999) Cancer Res. 59, 6999–6004
2. Chinnaiyan, A. M. (1999) Neoplasia 1, 1–15
3. Aritomi, M., Kunishima, N., Inohara, N., Ishibashi, Y., Ohta, S., and Morikawa, K. (1997) J. Biol. Chem. 272, 27886–27892
4. Boyd, M., Gable, G. R., Hargovan, S., Houghton, A. B., Malstrom, S., Avery, B. J., Ebb, R. G., Subramanian, T., Chittenden, T., Lutz, R. J., Chinnavarai, G. (1995) Oncogene 11, 1921–1928
5. Golgi, Z. P., Millman, C. L., and Korsmeyer, S. J. (1993) Cell 74, 609–619
6. Finucane, D. M., Bossy-Wetzel, E., Waterhouse, N. J., Cotter, T. G., and Green, D. R. (1999) J. Biol. Chem. 274, 2225–2233
7. Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L., Ng, S. L., and Fesik, S. W. (1996) Nature 381, 335–341
8. Schlesinger, P. H., Gross, A., Yin, X. M., Yamamoto, K., Saito, M., Waksman, G., and Korsmeyer, S. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11357–11362
9. Marzo, I., Brenner, C., Zamzami, N., Jurgenesmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C., and Kroemer, G. (1998) Science 281, 2027–2031
10. Matsuyama, S., Schendel, S. L., Xie, Z., and Reed, J. C. (1998) J. Biol. Chem. 273, 30995–31000
11. Antonsson, B., Conti, P., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J. J., Mazaee, G., Maundrell, K., Gamble, F., Sadoul, R., and Martinou, J. C. (1997) Science 277, 370–372
12. Jurgenesmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Broderson, D., and Reed, J. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4997–5002
13. Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., and Trautman, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14681–14686
14. Kharbanda, S., Pandey, P., Schod field, L., Israels, S., Roneskne, R., Yoshida, K., Bhati, A., Yuan, Z. M., Saxena, S., Weischelbraum, R., Nalin, C., and Kufe, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6939–6942
15. Mizuta, M., Stockwell, B. P., Steinhöfel, R., Salvesen, G. S., and Dixit, V. M. (1998) J. Biol. Chem. 273, 2926–2930
16. Salvesen, G. S., and Dixit, V. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10964–10967
17. Laemmli, U. K. (1971) Nature 227, 680–685
18. Dorward, D. W. (1993) Anal. Biochem. 209, 219–223
19. Vargus, C., McEwan, A. G., and Downie, J. A. (1993) Anal. Biochem. 209, 323–326
20. Shewach, D. S., Hahn, T. M., Chang, E., Hertle, L. W., and Lawrence, T. S. (1994) Cancer Res. 54, 3218–3223
21. Semino-Mora, C., Leon-Monzon, M., and Dalakas, M. C. (1997) Lab. Invest. 76, 487–495
22. Li, P., Nijahawan, D., Bujhaldark, I., Sinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
23. Zou, H., Li, Y., Liu, X., and Wang, X. (1999) J. Biol. Chem. 274, 11549–11556
24. Munn, R. A. (1974) Trends Cell Biol. 8, 324–330
25. Adachi, S., Cross, A. R., Babior, B. M., and Gottlieb, R. A. (1997) Trends Cell Biol. 7, 21878–21882
26. Li, P., Nijahawan, D., Budihardjo, I., Sinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
27. Zou, H., Li, Y., Liu, X., and Wang, X. (1999) J. Biol. Chem. 274, 11549–11556
28. Munn, R. A. (1974) Trends Cell Biol. 8, 324–330
29. Adachi, S., Cross, A. R., Babior, B. M., and Gottlieb, R. A. (1997) J. Biol. Chem. 272, 21878–21882
30. Krupner, A., Matsuo-Yagi, A., Gottlieb, R. A., and Babior, B. M. (1996) J. Biol. Chem. 271, 21629–21636

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