termined in total lysates by immunoblotting (*23*). For immunoprecipitations, cells were lysed in Nonidet P-40 isotonic lysis buffer (*23*) at 24 hours after transfection, and soluble lysates were incubated with either 1 μg/ml anti-HA (clone 12CA5, Boehringer Mannheim); 1 μg/ml rabbit anti-Myc (Santa Cruz), anti-FLAG (clone M2, Scientific Imaging Systems), or isotype-matched mouse IgG (anti-HA control); or normal rabbit serum (anti-Myc control) overnight at 4°C. Protein A–Sepharose 4B (Zymed Laboratories) was added [5% (v/v)] for an additional hour of incubation by rotation. Immune complexes were centrifuged and washed with excess cold Nonidet P-40 isotonic lysis buffer at least four times, separated on a 15% SDS-polyacrylamide gel, and immunoblotted with rabbit anti-Myc, anti-HA or anti-FLAG. The proteins were detected with an ECL system (Amersham).

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15. 293T cells were transfected with pcDNA3-HA–*ced-9*, pcDNA3-HA–*ced-9*ΔTM, pcDNA3-Myc–*ced-4S*, or pcDNA3-Flag–*gata-1* as described (*13*). At 24 hours after transfection, cells were incubated with anti-HA, anti-Myc (clone 9E10, Santa Cruz), anti-FLAG, or control mouse IgG for 1 hour at 23°C, and the labeling was visualized with fluorescein-conjugated goat anti-mouse IgG. After washing, the cells were mounted in Slowfade (Molecular Probes) and examined with a Bio-Rad MRC 600 scanning confocal microscope equipped with an argon-xenon laser (*24*).
16. D. Wu and G. Nuñez, unpublished results.
17. 293T cells (10^7) were transiently transfected with the indicated expression plasmids (10 μg). The cells were washed twice with phosphate-buffered saline and then incubated for 10 min in 1 ml of cold hypotonic buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg antipain, and leupeptin (0.3 μg/ml) as described (*25*). The swollen cells were lysed by 15 strokes in a tight-fitting Douncer homogenizer, and the samples were centrifuged at 1000*g* for 6 min to remove nuclei. The resulting supernatant was centrifuged for 1 hour at 100,000*g*. The supernatant portion of the latter centrifugation was the soluble cytosolic (C) fraction; the pellet was the membrane and cytoplasmic organelle (M) fraction. The proteins from the C and M fractions were dissolved in 1 ml of lysis buffer, and samples of equal size were analyzed by immunoblotting.
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Prevention of Apoptosis by Bcl-2: Release of Cytochrome c from Mitochondria Blocked

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Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria. Overexpression of Bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli. Cytosolic cytochrome c is necessary for the initiation of the apoptotic program, suggesting a possible connection between Bcl-2 and cytochrome c, which is normally located in the mitochondrial intermembrane space. Cells undergoing apoptosis were found to have an elevation of cytochrome c in the cytosol and a corresponding decrease in the mitochondria. Overexpression of Bcl-2 prevented the efflux of cytochrome c from the mitochondria and the initiation of apoptosis. Thus, one possible role of Bcl-2 in prevention of apoptosis is to block cytochrome c release from mitochondria.

Genetic studies of programmed cell death in *Caenorhabditis elegans* have identified two genes, *ced-9* and *ced-3*, that play important roles in regulating and executing apoptosis (*1*). *ced-9* and its mammalian counterpart *bcl-2* prevent cells from undergoing apoptosis (*2*, *3*). *ced-3*, which encodes a protease of the interleukin-1β–converting enzyme (ICE), or caspase-1, family, is required for apoptosis (*4*, *5*). The mammalian protein CPP32 (caspase-3) shares sequence similarity and substrate specificity with CED-3 (*6*).

CPP32 is a cytosolic protein that normally exists as a 32-kD inactive precursor. It is cleaved proteolytically into a 20-kD and a 10-kD active heterodimer in cells undergoing apoptosis (*7*). Activated CPP32 cleaves poly(adenosine diphosphate–ribose) polymerase (PARP) (*7*), sterol regulatory element–binding proteins (*8*), and several other cellular proteins (*9*). Bcl-2, which seems to work upstream of CPP32, prevents activation of CPP32 in response to staurosporine and other agents that cause apoptosis (*10*). The mechanism by which Bcl-2 regulates apoptosis is unknown; however, the location of Bcl-2 on the outer membrane of mitochondria raises the possibility that its function may be related to the function of mitochondria, which have been implicated in apoptosis (*11*–*13*).

We previously developed an in vitro assay in which cytosol from normally growing cells induces the activation of CPP32 and fragmentation of DNA after incubation with deoxyadenosine triphosphate (dATP) (*14*). Fractionation of this cytosol preparation revealed that cytochrome c, which was released into the cytosol during homogenization, is required for activation of CPP32 and DNA fragmentation (*14*). We also observed a marked increase of cytochrome c in the cytosol of cells undergoing apoptosis induced by staurosporine, a broad-range protein kinase inhibitor that induces apoptosis in a variety of cell types (*14*).

Cytochrome c is encoded by a nuclear gene and translated by cytosolic ribosomes as apopcytochrome c (*15*). Apocytochrome c is subsequently translocated into the mitochondria where a heme group is attached covalently to form holocytochrome c (*15*). The increase in cytosolic holocytochrome c upon apoptosis suggests that mitochondria may participate in apoptosis by releasing cytochrome c. This observation raises the possibility that Bcl-2, located on the outer membrane of mitochondria, may prevent cell death by blocking the release of cytochrome c. We tested this possibility in the following experiments.

Human acute myeloid leukemia (HL-60) cells were transfected with a retroviral vector containing a neomycin resistance gene (*neo* cells), or the same vector containing a cDNA encoding human Bcl-2 (Bcl-2 cells). The latter cells, which overexpress Bcl-2, resist apoptosis induced by clinically used anticancer drugs such as arabinosylcytosine (Ara-C), etoposide, and mitoxantrone hydrochloride (*16*). We isolated the mitochondria and cytosol from the two cell lines by differential centrifugation (*17*) and confirmed overexpression of Bcl-2 on the mitochondria by protein im-

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munoblot analysis using an antibody to Bcl-2 (Fig. 1A). Next, we treated cells from the two lines with 1 μM staurosporine for various lengths of time (*18*). Treatment of the control *neo* cells with staurosporine resulted in the activation of CPP32 in the cytosol, as indicated by the cleavage of PARP (Fig. 1B), whereas no PARP cleavage was detected in the cytosol of Bcl-2 cells. We then measured cytochrome c in the mitochondria (Fig. 1C) and cytosol (Fig. 1D) by protein immunoblot analysis. Without staurosporine treatment, most of the detectable cytochrome c in both cell lines was in the mitochondria. Cytochrome c in the cytosol of *neo* cells increased significantly after 1 hour of treatment with staurosporine, and it continued to increase for another 3 hours. The amount of cytochrome c in mitochondria showed a corresponding decrease, and cytochrome c became almost undetectable by the end of 4 hours of staurosporine treatment. In contrast, there was little change in cytochrome c in either the cytosol or mitochondria in the cells overexpressing Bcl-2.

Mitochondrial membrane depolarization is an early event of apoptosis, and overexpression of Bcl-2 prevents it (*13*, *19*). To see whether the release of cytochrome c from mitochondria is a consequence of depolarization, we treated samples of cells with staurosporine for various lengths of time and measured their mitochondrial membrane potential by staining with rhodamine 123 (*20*), a cationic fluorophore taken up by mitochondria as a result of their membrane potential. The rhodamine 123 uptake was visualized by laser scan confocal microscopy (*19*) (Fig. 1E). The *neo* cells treated with staurosporine for 2 hours (Fig. 1E, image c) and 4 hours (Fig. 1E, image d) showed no loss of mitochondrial membrane potential, but most mitochondrial cytochrome c had been released by 2 hours of treatment and was undetectable after 4 hours. The mitochondrial membrane potential of *neo* cells was eventually lost after 12 hours of staurosporine treatment (Fig. 1E, image e). Bcl-2 cells showed little change of mitochondrial membrane potential after 2, 4, and 12 hours of staurosporine treatment (Fig. 1E, images e, f, and g). This observation confirms the previous finding that Bcl-2 overexpression protects cells from losing their mitochondrial membrane potential (*13*, *19*). The release of cytochrome c does, however, precede the loss of mitochondrial membrane potential.

To test whether the release of cytochrome c into the cytosol is restricted to staurosporine-induced apoptosis, we tested another apoptosis-inducing reagent, etoposide, a widely used anticancer agent (*21*). Etoposide stabilizes covalent complexes between topoisomerase II and genomic DNA, resulting in DNA strand breaks (*21*). Bcl-2 inhibits etoposide-induced apoptosis without affecting the DNA strand breaks (*21*). Treatment of *neo* cells with 50 μM etoposide, a concentration that is attained clinically (*21*), resulted in the activation of caspases after about 3 hours, as indicated by the cleavage of PARP (Fig. 2A) and DNA fragmentation (Fig. 2C). Bcl-2 cells showed no activation of CPP32 or DNA fragmentation. Protein immunoblot analysis of cytosol from etoposide-treated *neo* cells showed a significant increase of cytochrome c as early as 1 hour, and this persisted for a total of 4 hours (Fig. 2B). In Bcl-2 cells, no cytosolic cytochrome c was detected up to 3 hours of etoposide treatment, but at 4

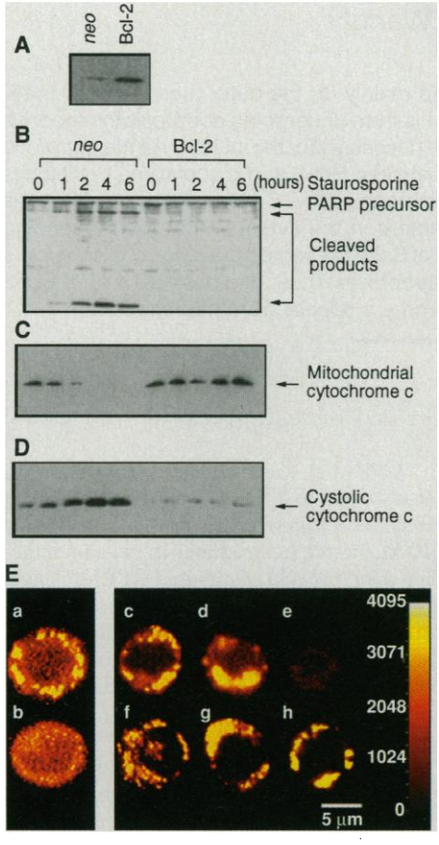

Fig. 1. Induction of cytochrome c release from the mitochondria to the cytosol by staurosporine. (**A**) Protein immunoblot analysis of Bcl-2 expression in mitochondria from HL-60 *neo* and Bcl-2 cells. Samples (25 μg) of mitochondria from *neo* and Bcl-2 cells were subjected to 15% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose filter. The filter was probed by an antibody to Bcl-2 (Santa Cruz), and the antigen-antibody complex was visualized by an enhanced chemiluminesence method (*10*). (**B**) Cleavage of in vitro–translated, ^{35}S-labeled human PARP by S-100 fractions from *neo* and Bcl-2 cells treated with staurosporine for the indicated amount of time. Human PARP was in vitro–translated and labeled with ^{35}S as described (*10*). S-100 fractions (5 μg) were incubated with translated PARP (5 μl) at 30°C for 5 min. The samples were subjected to 12% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to an x-ray film (Kodak) for 4 hours at room temperature. (**C**) Immunoblot analysis of cytochrome c in mitochondria isolated from *neo* and Bcl-2 cells treated with staurosporine for the amount of time indicated in (B). (**D**) Immunoblot analysis of cytochrome c in S-100 (cytosolic) fractions isolated from *neo* and Bcl-2 cells treated with staurosporine for the amount of time indicated in (B). Samples (25 μg) of mitochondria and the S-100 fraction from each time point were used for immunoblot analysis of cytochrome c as described in (*10*). (**E**) Mitochondrial membrane potential of staurosporine-treated *neo* and Bcl-2 cells were measured by rhodamine 123 staining and visualized by laser scan confocal microscopy (*29*). Image a, *neo* cell without staurosporine treatment; image b, *neo* cell treated with carbonyl cyanide *m*-chlorophenylhydrazone for 10 min to disrupt their membrane potential; image c, *neo* cell treated with staurosporine for 2 hours; image d, *neo* cell treated with staurosporine for 4 hours; image e, *neo* cell treated with staurosporine for 12 hours; image f, Bcl-2 cell treated with staurosporine for 2 hours; image g, Bcl-2 cell treated with staurosporine for 4 hours; image h, Bcl-2 cell treated with staurosporine for 12 hours.

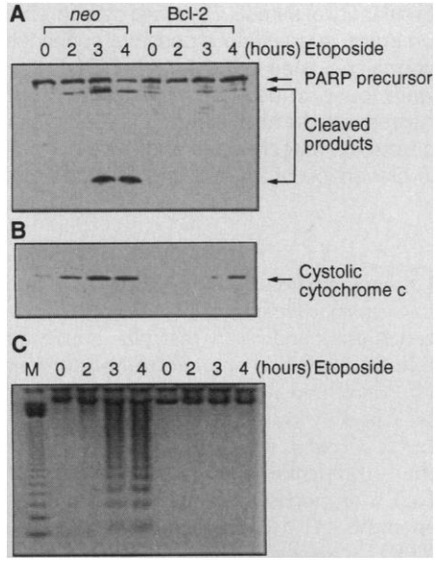

Fig. 2. Induction of cytochrome c release into the cytosol by etoposide. (**A**) Cleavage of in vitro–translated, ^{35}S-labeled human PARP by S-100 fractions from *neo* and Bcl-2 cells treated with 50 μM etoposide for the indicated amount of time. (**B**) Immunoblot analysis of cytochrome c in the S-100 fraction isolated from *neo* and Bcl-2 cells treated with etoposide for the time indicated in (A). (**C**) DNA fragmentation assay of *neo* and Bcl-2 cells treated with etoposide for the indicated amount of time. The DNA fragmentation assay was done as described (*20*).

hours, a small amount was observed.

These experiments show that cytochrome c is released from mitochondria early in apoptosis before mitochondrial depolarization, activation of caspases, and DNA fragmentation. Overexpression of Bcl-2 prevents the release of cytochrome c from the mitochondria to the cytosol. Similar data were obtained with the cells overexpressing another member of the Bcl-2 family, Bcl-x_L (*22*).

The prevention of release of cytochrome c from mitochondria in cells overexpressing Bcl-2 was also apparent during in vitro incubation of mitochondria in hypotonic buffer (Fig. 3). We isolated mitochondria from large-scale cultures of *neo* and Bcl-2 cells. Similar amounts of mitochondria from the two cell lines, as determined by protein concentration and confirmed by oligomycin-sensitive mitochondrial adenosine triphosphatase activity (*23*), were incubated in vitro with a buffer solution containing 150 mM sucrose at 30°C for 1 hour. The mitochondria were then pelleted by centrifugation, and the presence of cytochrome c in the supernatants and mitochondrial pellets was detected by protein immunoblot analysis (Fig. 3A). A reduction in sucrose concentration from 250 mM, in which the isolated mitochondria were originally resuspended, to 150 mM and incubation at 30°C resulted in the release of cytochrome c from the mitochondria of the *neo* cells but not from the Bcl-2 cells. Consistent with this finding, the mitochondrial pellet from the Bcl-2 cells retained much more cytochrome c than that of the *neo* cells after the same period of incubation.

The S-100 fraction of cytosol prepared by Dounce homogenization of HeLa cells in a hypotonic buffer without sucrose contains cytochrome c released from mitochondria during homogenization (*14*) (Fig. 3A). The cytochrome c in the S-100 fraction can be immunodepleted with a monoclonal antibody to cytochrome c [designated S-100 (-cyt. c)] (Fig. 3A). The S-100(-cyt. c) fraction lost its ability to initiate the apoptotic program on the addition of dATP, as measured by the cleavage of CPP32, and apoptotic activity was restored by the addition of purified cytochrome c (Fig. 3B).

This immunodepletion and reconstitution system allowed us to test directly whether mitochondria trigger the cytosolic apoptotic program by releasing cytochrome c and whether overexpression of Bcl-2 blocks this process. Mitochondria from *neo* and Bcl-2 cells were incubated in a low-sucrose buffer solution in vitro for 1 hour (Fig. 3B). The mitochondria were then pelleted, and the resulting supernatants were incubated with immunodepleted HeLa cell S-100(-cyt. c) fraction and ^{35}S-labeled CPP32. The CPP32 was cleaved when the S-100(-cyt. c) fraction was incubated with the supernatant from mitochondria of *neo* cells in the presence of dATP but not with that of Bcl-2 cells. The mitochondrial supernatants alone were not able to activate CPP32, which is consistent with our previous finding that additional cytosolic factors are required for the dATP- and cytochrome c–dependent activation of CPP32 (*14*). Activation of CPP32 was prevented by the inclusion of a monoclonal antibody to cytochrome c [immunoglobulin G2A (IgG2A)], but not by a control monoclonal IgG2A, showing the specificity of cytochrome c in activating CPP32.

Cytochrome c is translated by cytosolic ribosomes as apocytochrome c, and it is assembled in the mitochondria into holocytochrome c (*15*). We investigated which forms support apoptosis. We prepared apocytochrome c from bovine cytochrome c (*24*) and found that holocytochrome c, but not apocytochrome c, reconstituted the CPP32 activation activity with the S-100 (-cyt. c) fraction (Fig. 4). This result is consistent with the idea that the release of cytochrome c from mitochondria, not the block of importation of cytochrome c, may lead to apoptosis. Taken together, these data support a model in which Bcl-2, located on the outer membrane of mitochondria, prevents the initiation of the cellular apoptotic program by preventing the release of cytochrome c from mitochondria.

The mechanism by which cytochrome c is released from mitochondria remains to be determined. However, the release of cytochrome c from mitochondria seems to represent a pathway of apoptosis distinct in several ways from the one reported by Kroemer and co-workers who showed that a 50-kD protein, released from mitochondria upon mitochondrial depolarization, is able to induce chromatin condensation and DNA fragmentation when incubated with nuclei (*19*). First, cytochrome c release precedes mitochondrial membrane depolarization (Fig. 1, C to E); second, cytosolic cytochrome c participates in activating CPP32 (*14*), whereas the 50-kD mitochondrial factor directly induces apoptotic changes in the nuclei (*19*); and finally, activated CPP32 induces apoptotic changes in nuclei only in the presence of cytosol, and such changes can be blocked by the

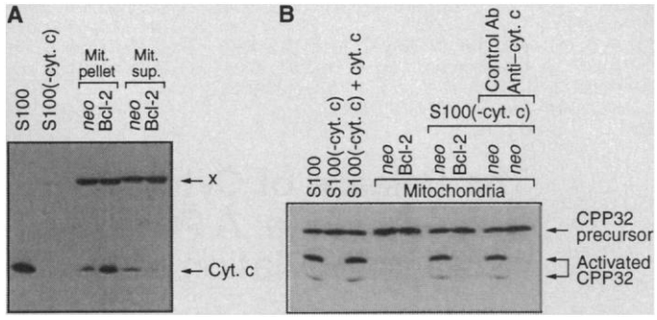

Fig. 3. In vitro reconstitution of the CPP32 activation reaction by proteins released from mitochondria. (**A**) Protein immunoblot analysis of cytochrome c from samples (25 μg) of the S-100 fraction from HeLa cells (S-100), the S-100 fraction immunodepleted of cytochrome c [S-100 (-cyt. c)], or the supernatants (sup.) and pellets of mitochondria (Mit.) (10 μg) from *neo* or Bcl-2 cells incubated at 30°C for 1 hour with 150 mM sucrose and then centrifuged. X denotes a cross-reacting protein with this antibody. (**B**) CPP32 activation reactions were carried out by incubating samples (3 μl) of in vitro–translated and affinity-purified CPP32 (*10*) with HeLa S-100 fraction (S-100), S-100(-cyt. c), S-100(-cyt. c) plus 0.2 μg of human cytochrome c [S-100(-cyt. c) + cyt. c], the supernatants of the incubated mitochondria alone, the supernatants plus S-100(-cyt. c), the supernatants supplemented with S-100(-cyt. c) plus 0.5 μg of antibody to cytochrome c (anti-cyt. c) (IgG2A), or 0.5 μg of control IgG2A (control Ab) (antibody to SLA, major histocompatibility complex class I, American Type Culture Collection CRL-1945) in addition to 1 mM $MgCl_2$ and 1 mM dATP in 25 ml of buffer A. After incubation at 30°C for 1 hour, the samples were subjected to 15% SDS-PAGE and transferred to nitrocellulose filters. The filters were exposed to x-ray film (Kodak) for 4 hours at room temperature.

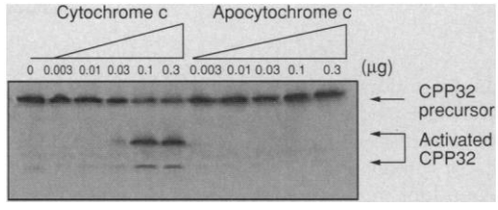

Fig. 4. Reconstitution of CPP32 activation by cytochrome c but not by apocytochrome c. Bovine cytochrome c (Sigma) was purified further through a Mono S column, and the heme group of cytochrome c was removed as described (*24*). The apocytochrome c and cytochrome c were dialyzed against buffer A before use. Samples (25 μg) of HeLa S-100 fraction immunodepleted of cytochrome c were supplemented with increasing amounts of apocytochrome c or cytochrome c as indicated and used for the CPP32 activation reaction as described in Fig. 3.

presence of a submicromolar concentration of CPP32-specific tetrapeptide inhibitor (*25*). On the other hand, the 50-kD factor, once released from mitochondria upon depolarization, functions without cytosol and is insensitive to the CPP32 inhibitor (*13*). Despite these differences, it is possible that these two pathways may work together to induce complete apoptosis, in which case Bcl-2 must block both pathways.

Other factors that work together with cytochrome c to activate CPP32 and subsequent DNA fragmentation appear to be of cytosolic origin and present in similar amounts in the cytosol from both *neo* and Bcl-2 cells (*26*). The molecular identity of these factors remains to be determined.

The mechanism by which Bcl-2 blocks the release of protein from mitochondria and the regulation of this process are topics of future study. Especially in the case of cytochrome c, the release appears to be independent of any noticeable structural changes in the mitochondria. The recent determination of the nuclear magnetic resonance and crystal structure of Bcl-x_L (*27*), and the demonstration of phosphorylation of Bcl-2 and its pro-apoptotic family member BAD (*28*), may also shed some light on the function and regulation of this family of proteins. The arrangement of the α helices in Bcl-x_L is reminiscent of the membrane translocation domain of bacterial toxins, in particular, diphtheria toxin and colicins. Inasmuch as the diphtheria toxin translocation domain is thought to form a membrane pore (*27*), the Bcl-2 family of proteins could also be part of a pore structure that might control the release of cytochrome c. The pro- and anti-apoptotic regulation mechanisms, such as the sequestration of phosphorylated BAD in the cytosol or the association of Raf with Bcl-2 (*28*), might assert their influences on apoptosis by regulating the permeability of such a pore.

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17. HL-60 cells overexpressing Bcl-2 and the vector control cells were harvested by centrifugation at 600*g* for 10 min at 4°C. The cell pellets were washed once with ice-cold phosphate-buffered saline (PBS) and resuspended with five volumes of buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose. The cells were homogenized with 10 strokes of a Teflon homogenizer, and the homogenates were centrifuged twice at 750*g* for 10 min at 4°C. The supernatants were centrifuged at 10,000*g* for 15 min at 4°C, and the resulting mitochondria pellets were resuspended in buffer A containing 250 mM sucrose and frozen in multiple samples at −80°C. The supernatants of the 10,000*g* spin were further centrifuged at 100,000*g* for 1 hour at 4°C, and the resulting supernatants (designated S-100) were divided into samples and frozen at −80°C for further experiments.
18. On day 0, the cells (~2.5×10^5 cells/ml) were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, penicillin (100 μg/ml), and streptomycin (100 μg/ml) and incubated at 37°C in a 5% CO_2 incubator. On day 2, cells were treated either with staurosporine (added to a final concentration of 1 μM) for 0, 1, 2, 4, and 6 hours, or with etoposide (added to a final concentration of 50 μM) for 0, 2, 3, and 4 hours. The S-100 supernatants and mitochondria from these treated cells were prepared as described in (*17*).
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29. *neo* and Bcl-2 cells were set up and treated with staurosporine as described in (*17*). After centrifugation at 600*g* for 10 min at 4°C, the cell pellets were resuspended in PBS containing 5 μM rhodamine 123 (Sigma) and incubated at 37°C for 15 min. The cell suspension was centrifuged in a microcentrifuge for 30 s, and the pellet was resuspended in 20 μl of PBS, plated onto a 25-mm round glass cover slip coated with poly-D-lysine, and mounted into a perfusion chamber for confocal imaging. The fluorescence images were collected with a Meridian Insight-Point Laser scanning confocal microscope (Meridian Instrument) equipped with a Zeiss Axioplan microscope. The objective lens was a 100× numerical aperture 1.4 PlanApo lens. The aperture size of the pinhole was 10 to 40 μm. Confocal optical sections were estimated to be less than 1 μm in thickness. Cells were excited with the 488-nm line of an argon laser, and emitted fluorescence was detected through a 530/30 band-pass filter with an intensified, cooled charge-coupled-device camera. A typical cell from a population of ~100 was presented.

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The Release of Cytochrome c from Mitochondria: A Primary Site for Bcl-2 Regulation of Apoptosis

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In a cell-free apoptosis system, mitochondria spontaneously released cytochrome c, which activated DEVD-specific caspases, leading to fodrin cleavage and apoptotic nuclear morphology. Bcl-2 acted in situ on mitochondria to prevent the release of cytochrome c and thus caspase activation. During apoptosis in intact cells, cytochrome c translocation was similarly blocked by Bcl-2 but not by a caspase inhibitor, zVAD-fmk. In vitro, exogenous cytochrome c bypassed the inhibitory effect of Bcl-2. Cytochrome c release was unaccompanied by changes in mitochondrial membrane potential. Thus, Bcl-2 acts to inhibit cytochrome c translocation, thereby blocking caspase activation and the apoptotic process.

Bcl-2 and its relatives (for example, Bcl-x, E1B 19K, and CED-9) are potent inhibitors of apoptotic cell death (*1–3*). Bcl-2 is located predominantly in the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear membrane (*4–8*), and it appears to prevent apoptosis at a point in the process upstream of the activation of CED-3 family proteases such as caspase-3 (CPP32) (*9–12*). How Bcl-2 prevents protease activation is not known.

We used a cell-free system based on *Xenopus* egg extracts in which recombinant Bcl-2 prevents protease activation and subsequent apoptotic effects (*13–16*). This system is similar to other cell-free systems