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

based on cytosol derived from apoptotic cultured cells (*14*, *17–20*), but with one important exception: the spontaneous apoptosis in the *Xenopus* system depends on the presence of a heavy membrane fraction enriched in mitochondria (*13*). These organelles, when incubated in *Xenopus* egg cytosol, generate a soluble proteinaceous factor that can induce apoptosis rapidly in mitochondria-free extracts (*16*). Cytochrome c was recently identified as such a factor—it is released from mitochondria in apoptotic cells and can trigger activation of DEVD-specific caspases (DEVDases) and apoptotic effects in cell-free systems containing cytosol (*16*, *21*).

We therefore examined the effect of exogenous Bcl-2 on cytochrome c release. Incubation of *Xenopus* egg mitochondria in the cell-free system resulted in the time- and temperature-dependent release of cytochrome c into the soluble fraction (Fig. 1, A and C). This release, which required the presence of cytosol (*16*), occurred after a lag period (~3 hours) corresponding to the time when DEVD-specific protease activity first appeared, which would be expected if cytochrome c is required for protease activation. About 1 hour later, apoptotic figures began to be observed in the added nuclei. Spectrophotometric analysis of the released cytochrome c (*16*) confirmed that it was the holoprotein, and because import into the mitochondria is required for the attachment of the heme group (*22*, *23*), our results support the idea that cytochrome c is actually released and not accumulated in the cytosol as a result of failed import. In addition, the presence of cycloheximide in our extracts precludes the synthesis of apocytochrome c, and immunoblotting confirmed the near absence of cytochrome c in egg cytosol incubated alone (Fig. 1, A and C).

When baculovirus-expressed Bcl-2 was added to extracts containing mitochondria, cytochrome c release was blocked (Fig. 1B). Accompanying apoptotic features of nuclear disintegration (Fig. 1, B and D), protease activation (Fig. 1B) and the cleavage of fodrin (Fig. 1E) (*24*) were also prevented. The addition of control Sf9 cell lysate containing expressed β-galactosidase had no effect. Thus, Bcl-2 blocks apoptosis in the *Xenopus* system by inhibiting the release of cytochrome c from mitochondria.

We next examined whether the ability of Bcl-2 to block cytochrome c release in vitro could also be observed in cells. CEM cells were treated with different agents to induce apoptosis, and the effects of ectopic expression of Bcl-2 on cytochrome c translocation were assessed (Fig. 2). Several agents capable of eliciting apoptosis all induced the appearance of cytochrome c in cytosol (Fig. 2A). Expression of Bcl-2 inhibited apoptosis and prevented the release of cytochrome c (Fig. 2B). The appearance of cytosolic cytochrome c was correlated with a loss of the proform of CPP32 and the appearance of protease activity, as determined by the cleavage of fodrin (*24*). In contrast, the caspase inhibitor zVAD-fmk had no effect on the translocation of cytochrome c to the cytosol, although it was able to block apoptosis, the loss of proCPP32, and fodrin cleavage (Fig. 2C) as described (*25*). By confocal immunofluorescence microscopy (*26*), cytochrome c displayed a punctate localization in untreated HeLa cells, consistent with its location within mitochondria. After the induction of apoptosis by ultraviolet-B (UVB) irradiation, the immunostaining pattern became more diffuse, consistent with a translocation of cytochrome c into the cytosol. This

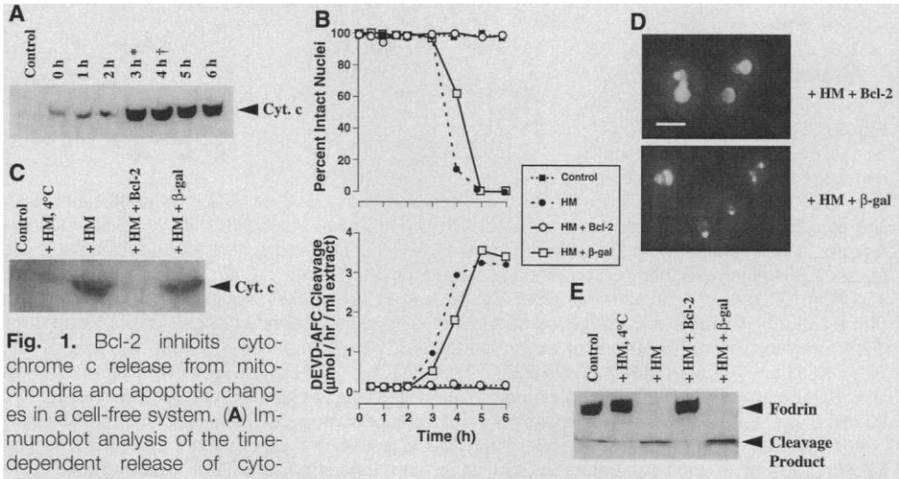

Fig. 1. Bcl-2 inhibits cytochrome c release from mitochondria and apoptotic changes in a cell-free system. (**A**) Immunoblot analysis of the time-dependent release of cytochrome c (Cyt. c) from mitochondria into the cytosol in the cell-free apoptosis system. Mitochondria-depleted *Xenopus* egg extract was reconstituted by the combination of cytosol, light membrane (predominantly endoplasmic reticulum and nuclear membrane precursor vesicles), and rat liver nuclei (*13*). [In all experiments, cytosol was supplemented with an adenosine triphosphate (ATP) regenerating system consisting of 10 mM phosphocreatine, 2 mM ATP, and creatine phosphokinase (150 mg/ml)]. Mitochondria (HM, 2% v/v) were added and the mixture incubated at 22°C. At the times indicated (in hours), cytosolic cytochrome c content was analyzed after first pelleting the membranes (12,000*g*) and filtering the supernatant through 0.2-μm and then 0.1-μm Ultrafree-MC filters (Millipore). The resulting cytosolic protein (10 μl, ~300 μg of protein) was separated by SDS–polyacrylamide gel electrophoresis (PAGE) (12% gel) followed by transfer to polyvinylidene difluoride (PVDF) membrane. The blot was probed with a monoclonal antibody to horse cytochrome c peptide (7H8.2C12) used at a dilution of 1:500. A secondary probe with horseradish peroxidase–labeled antibodies (Amersham) was detected by ECL (Amersham). DEVD-specific protease activation and nuclear apoptosis were assessed as described in (B). The asterisk indicates the onset of DEVD-specific protease activation, and the dagger indicates the onset of nuclear apoptosis and full protease activation. (**B**) Bcl-2 blocks apoptosis induced by the presence of HM. Extracts reconstituted with or without HM (2% v/v) were incubated (22°C) with or without lysate (1.8% v/v) from baculovirus-infected Sf9 cells expressing either Bcl-2 or β-galactosidase (β-gal) (*13*). At the indicated times, samples were assessed for percent intact rat liver nuclei (*13*) (top) and DEVD-specific protease activation (bottom). DEVD-specific protease was measured by incubating extract (2 μl) with z-DEVD-AFC (80 μM, Enzyme Systems Products, Dublin, California) in 100 μl of buffer A [250 mM sucrose, 20 mM Hepes-KOH, pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT)]. At 15 min the reaction was stopped by the addition of 1% sodium acetate trihydrate in 175 mM acetic acid (50 μl) (*50*) and placed on ice. Fluorescence (400 to 505 nm) after the addition of water (1 ml) was compared with a standard curve of 7-amino-4-trifluoromethyl-coumarin (AFC, Sigma). Panels (C), (D), and (E) and Fig. 3, A and B, also relate to this experiment, which is representative of three independent experiments. A similar activation of zYVAD-AFC (Enzyme Systems Products) cleaving activity was not seen (*26*), arguing that DEVDase is more similar to caspase-3 (CPP32) than caspase-1 (ICE). (**C**) Bcl-2 blocks cytochrome c release from mitochondria. Cytosolic cytochrome c content in end point (6 hours) extract samples from which HM had been removed was analyzed by protein immunoblot. A rabbit antiserum raised to bovine and tuna heart cytochrome c (Sigma) cross-reacted with *Xenopus* egg cytochrome c and was used (at 1:500) as the primary antibody. (**D**) Apoptotic nuclear morphology is prevented by Bcl-2. Photomicrographs of Hoechst 33258-stained rat liver nuclei were taken at 6 hours. Chromatin condensation and nuclear fragmentation typical of apoptosis (bottom panel) is absent in rat liver nuclei incubated in extracts containing Bcl-2. The bar is 20 μm. (**E**) Fodrin cleavage induced by HM is prevented by Bcl-2. End point (6 hours) unfiltered extract samples (4 μl) were analyzed by immunoblot with a monoclonal antibody (Chemicon International, 1:1000) to fodrin (nonerythroid spectrin).

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change in distribution was prevented by Bcl-2 expression, but not by treatment with zVAD-fmk.

We next examined whether Bcl-2 might also inhibit the apoptosis-inducing effect of cytosolic cytochrome c. *Xenopus* egg extracts depleted of mitochondria lack cytochrome c and fail to activate endogenous DEVDases; however, the addition of purified cytochrome c causes rapid DEVDase activation, which in turn leads to downstream apoptotic effects (*16*). Bcl-2 effectively inhibited mitochondria-dependent apoptosis (Fig. 3A) but could not block the activation of nuclear apoptosis (Fig. 3A), DEVDase (Fig. 3A), and fodrin cleavage (Fig. 3B) induced by exogenous cytochrome c. A careful titration showed that Bcl-2 could not reverse the effects of cytochrome c at any concentration, either in the presence (Fig. 3C) or absence (*26*) of mitochondrial membranes.

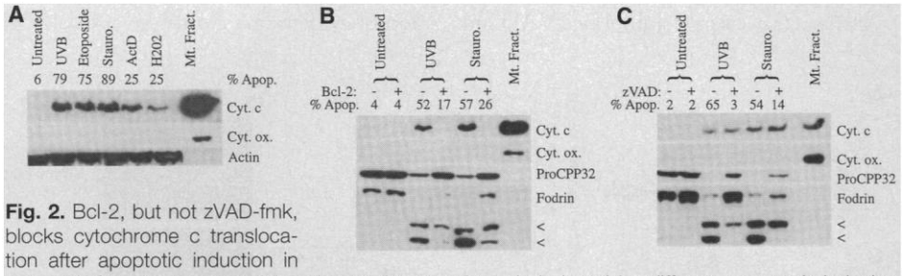

Fig. 2. Bcl-2, but not zVAD-fmk, blocks cytochrome c translocation after apoptotic induction in cells. (**A**) Cytochrome c translocation to the cytoplasm is induced by different apoptosis-inducing agents. CEM cells were treated as indicated with UVB (5 min), etoposide (100 μM), staurosporine (Stauro., 1 μM), actinomycin D (ActD, 1 μM), or H_2O_2 (400 μM) and cultured an additional 7 hours. Cytosolic extracts were then prepared and assessed for cytochrome c (Cyt. c), cytochrome c oxidase subunit II (Cyt. ox.), or actin by immunoblot. Cyt. ox. serves as a marker of mitochondrial contamination of the extracts. Extracts were prepared as follows. Cells were washed in phosphate-buffered saline (PBS) and suspended in 500 μl of extraction buffer [220 mM mannitol, 68 mM sucrose, 50 mM Pipes-KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (Chocktail, Boehringer Mannheim)]. After 30 min on ice, cells were lysed with a glass Dounce homogenizer, with 40 strokes of the B pestle. After centrifugation at 14,000*g* for 15 min, 50 μg of cytosolic protein or 5 μg of a mouse liver mitochondrial fraction (Mt. Fract.) were separated by SDS-PAGE and transferred to a PVDF membrane (for cytochrome c or CPP32) or Hybond-ECL-nitrocellulose membrane (for other proteins). (**B**) Cytochrome c translocation and apoptosis are inhibited in CEM cells by Bcl-2. CEM or CEM-Bcl-2 cells were treated with UVB (5 min) or staurosporine (500 nM), cultured an additional 6.5 hours, and cytosolic extracts assessed by immunoblot. (**C**) Apoptosis, but not cytochrome c translocation, is inhibited by zVAD-fmk. CEM cells were treated for 2 hours with 100 μM zVAD-fmk (Kamiya, Thousand Oaks, California), then treated with UVB (5 min) or staurosporine (1 μM) and cultured for 5 hours. Apoptosis was assayed in (A) to (C) by annexin-V–fluorescein isothiocyanate (FITC) staining as described (*51*). Arrows in (B) and (C) indicate cleavage products of fodrin.

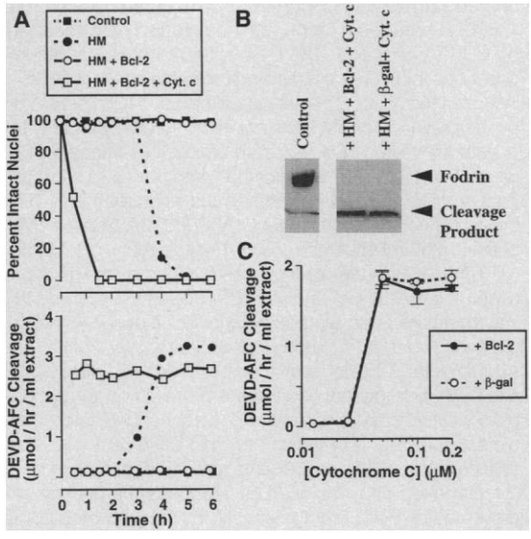

Fig. 3. Cytochrome c bypasses the inhibitory effect of Bcl-2 to induce apoptosis. (**A**) Cytochrome c rapidly induced nuclear apoptotic changes (top) and DEVD-specific protease activation (bottom) despite the presence of Bcl-2. Horse heart cytochrome c (0.05 μM) was added to extracts containing Bcl-2 at a concentration which was capable of blocking HM-induced apoptosis (Fig. 1). (**B**) Cytochrome c–induced fodrin cleavage, even in the presence of Bcl-2. See Fig. 1E for experimental details. (**C**) Bcl-2 did not reverse the effects of cytochrome c at any concentration. Horse heart cytochrome c was incubated in the presence of either β-gal– or Bcl-2–containing Sf9 cell lysate in a simplified extract mix of cytosol and HM (1% v/v). DEVD-specific protease activity measured at 1 hour showed β-gal and Bcl-2 lysates to have equivalent effects at all cytochrome c concentrations. Bcl-2 was added at a concentration shown to delay HM-induced protease activation by 2 hours (*26*). The data are mean and SEM of triplicates. The experiment is representative of two independent experiments.

These results showed that cytochrome c release from mitochondria is a critical step in the apoptotic process in this system and that Bcl-2 acts upstream of this event. It was still conceivable, however, that Bcl-2 acts on undefined cytoplasmic events leading to this release rather than on the mitochondrion itself. To address this possibility, we used a simplified cell-free system, consisting only of cytosol and the mitochondria-rich fraction (Fig. 4). Although this minimal system lacks endoplasmic reticulum (ER) and nuclei, it displays the same activation of endogenous DEVDase and fodrin cleavage seen in the complete extract (*16*). In this minimal system, as in the complete extracts, Bcl-2 blocked the activation of DEVDase (Fig. 4, A and C). To determine if this inhibition was due to mitochondrial association of Bcl-2, we first incubated mitochondria (HM) in the presence of baculovirus-expressed Bcl-2. The organelles were then pelleted and either resuspended in fresh cytosol or put back in the cytosol from which they had just come. As shown in Fig. 4A, the mitochondria that had been incubated with Bcl-2 could not activate DEVD-specific protease activity when placed in fresh cytosol lacking Bcl-2 or in the original cytosol. In contrast, untreated heavy membranes were able to activate protease when added to the cytosol supernatant removed from the Bcl-2–treated heavy membranes. We conclude that, in this system, Bcl-2 acts in association with the mitochondrial fraction. Again, Bcl-2 inhibited cytochrome c release in all cases where protease activation was blocked (Fig. 4B).

To examine whether Bcl-2 acts directly on the mitochondria, we performed an experiment in which half of the mitochondria were first incubated with Bcl-2. After this incubation, the other half of the mitochondria were added. We reasoned that this latter portion would not have access to the Bcl-2 that had associated with the former half. If Bcl-2 acts globally to inhibit a step upstream of the involvement of mitochondria, then Bcl-2 should inhibit protease activation even if concentrated in only a portion of the mitochondria. However, this was not observed. Rather, Bcl-2 inhibited cytochrome c release and the apoptotic effects when added to all of the mitochondria but not when incubated first in only half of them (Fig. 4, C and D). Thus, in this system Bcl-2 counters apoptosis by acting directly on mitochondria, at or near the mechanism responsible for cytochrome c release.

A loss in mitochondrial membrane potential ($\Delta\Psi$) has been correlated with the induction of apoptosis, and Bcl-2 protects mitochondria from this effect (*27–29*). Mitochondrial depolarization in apoptosis ap-

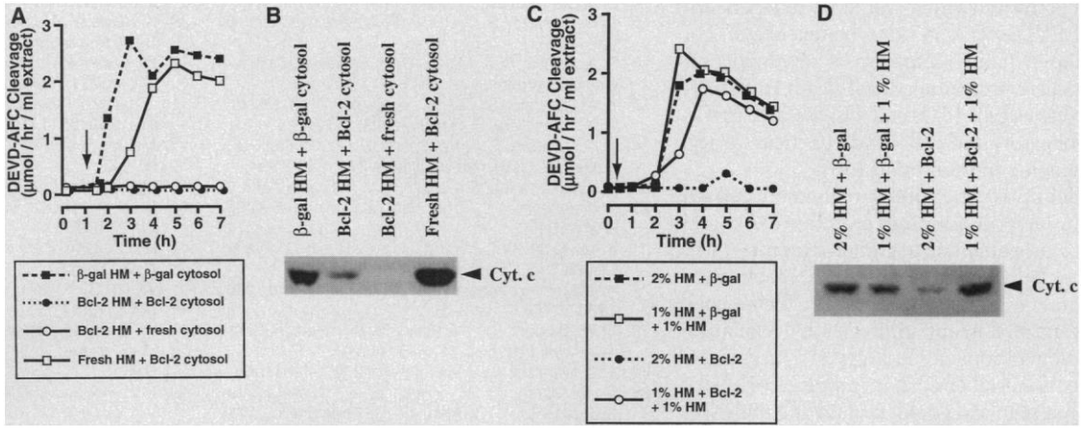

Fig. 4. Bcl-2 regulates apoptosis by acting in situ on mitochondria. (**A**) Bcl-2 acts at mitochondria to block apoptosis. Samples containing cytosol, HM (1% v/v), and Bcl-2 Sf9 cell lysate (2% v/v) were incubated for 1 hour (indicated by arrow) before separation and remixing of the cytosolic and HM fractions with fresh cytosol or HM. Fractions were separated by centrifugation (12,000*g*, 3 min, 22°C), the pellet resuspended in 1.5× volume of fresh cytosol, then repelleted(12,000*g*, 3 min, 22°C) and the Bcl-2–incubated fractions recombined as indicated with equivalent volumes of fresh cytosol or HM before further incubation to 7 hours. A control sample incubated with β-gal Sf9 lysate is also shown. The experiment is representative of two independent experiments. (**B**) Immunoblot of cytochrome c content in cytosol. Extracts from the experiment in Fig. 3A were sampled (6 hours) and assayed for cytochrome c content as described (Fig. 1A). (**C**) Bcl-2 acts in situ on mitochondria to block apoptosis. β-Gal or Bcl-2 Sf9 lysate was added to cytosol containing HM at either the full amount (2% v/v) or half of this amount and incubated at 22°C. At 10 min (arrow), more HM (the remaining half of the total) was added to the two samples indicated, before reincubation to 7 hours. This experiment is representative of three independent experiments. (**D**) Immunoblot of cytochrome c content in cytosol. Extracts from the experiment in Fig. 3C were sampled at 5 hours and assayed for cytochrome c content as described (Fig. 1A).

pears to be associated with the permeability transition (PT), an event involving the formation of a nonspecific channel in the mitochondrial membranes (*30*), which is also blocked by Bcl-2 (*31–34*).

To assess whether changes in mitochondrial $\Delta\Psi$ play a role in the *Xenopus* cell-free system, we measured the mitochondrial uptake of a membrane potential–sensitive dye, DiOC6(3), in extracts reconstituted from cytosol and mitochondria. DiOC6(3) uptake remained essentially unchanged during the course of apoptosis, regardless of whether Bcl-2 was present (Fig. 5A). [The small initial decline in DiOC6(3) uptake seen here did not occur in unfractionated extracts (*26*) and seems therefore to reflect an equilibration of the mitochondria when brought from buffer into cytosol.] This argues that changes in mitochondrial $\Delta\Psi$ do not accompany apoptosis in this system and, moreover, that Bcl-2 does not inhibit cytochrome c release only by protecting against mitochondrial depolarization. The protonophore CCCP (10 μM) caused a loss of dye content when added to mitochondria either before (*26*) or after cytochrome c release (Fig. 5B), showing that the mitochondrial membrane remained polarized throughout the process leading to cytochrome c translocation. As described (*13*), CCCP (10 μM) did not accelerate, but rather delayed, apoptotic events somewhat. CCCP could not reverse the anti-apoptotic effect of Bcl-2 (*26*); conversely, Bcl-2 could not overcome the effect of CCCP on DiOC6(3) retention (Fig. 5B). In the *Xenopus* system, then, the release of cytochrome c and its inhibition by Bcl-2 are unaccompanied by changes in $\Delta\Psi$ and thus seem not to involve a mitochondrial PT.

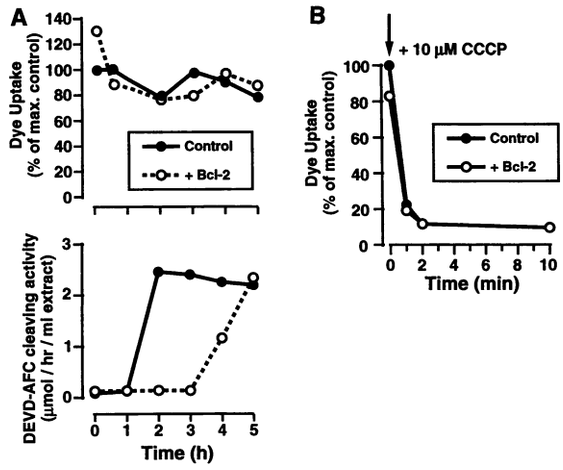

Fig. 5. Apoptosis in the *Xenopus* system is unaccompanied by mitochondrial depolarization, and Bcl-2 has no effect on mitochondrial membrane potential. (**A**) Mitochondrial uptake of DiOC6(3) is unchanged during apoptosis. Samples containing cytosol and HM (1.4% v/v) were incubated for 5 hours, with or without Bcl-2 Sf9 lysate (2% v/v). At the indicated times, a sample (20 μl) was removed, combined with 100 nM DiOC6(3) [Molecular Probes; 2 μl of a 1 μM stock in dimethyl sulfoxide (DMSO)] and further incubated for 10 min at 22°C. Samples were then diluted in 1 ml of buffer A and filtered through a 52-μm Nitex mesh and incubated for another 10 min before flow cytometric analysis (FacsCalibur, Becton Dickinson) of DiOC6(3) content of individual mitochondria. This last incubation had no effect on dye retention by the mitochondria (*26*). This experiment was representative of three separate experiments, except that in this case Bcl-2 did not completely block apoptosis, but delayed it by ~2 hours, as indicated by DEVD-AFC cleaving activity. (**B**) The protonophore CCCP decreases dye content in mitochondria even after cytochrome c release has occurred. To the 3-hour samples from (A), 10 μM CCCP was added, and DiOC6(3) retention was again measured at the indicated times.

These results are fundamentally different from those reported by Susin *et al.*, who used a cell-free system lacking cytosol but containing mitochondria and nuclei suspended in buffer (*35*). In their system, mitochondria that have undergone the PT release apoptosis-inducing factor (AIF), a 50-kD protease that can act directly on resuspended nuclei to induce DNA fragmentation and chromatin condensation. These effects of AIF are insensitive to *N*-acetyl-DEVD-CHO, an inhibitor of CPP32-like caspases. There may be multiple mechanisms through which mitochondria can signal cell death, and perhaps AIF and cytochrome c participate in parallel or alternative apoptotic pathways. However, the involvement of AIF can be ruled out in our system, and in at least some other cytosol-based cell-free systems, because all of the events observed, including chromatin condensation and nuclear disintegration, are effectively blocked by *N*-acetyl-DEVD-CHO (*16, 36*). Furthermore, in some instances of apoptosis in living organisms, an

AIF pathway (which would be independent of DEVDases) can also be excluded. For example, CED-3 protease is absolutely required for programmed cell death in *Caenorhabditis elegans* (*37*), and caspase-3 (CPP32) is obligatory for cell death in areas of the developing murine brain (*38*).

Bcl-x_L is structurally related to certain bacterial pore-forming proteins (*39*), and Bcl-2 probably has a similar structure (*40*). Thus, Bcl-2 could conceivably block cytochrome c efflux directly. Or, Bcl-2 may prevent this event indirectly by regulating the flow of ions, including Ca^{2+}, across the mitochondrial and ER membranes (*41–46*). Such a scenario could explain the ability of Bcl-2, in some cell types, to block apoptosis even when its location is restricted to the ER (*47*). In the cell-free system described here, ER membranes are not required for activation of DEVD-specific proteases (Fig. 4), and thus a possible effect of Bcl-2 on this compartment may not be discernible.

Our results show that Bcl-2 acts on the mitochondria with which it is associated (Fig. 4). This localized action of Bcl-2 may depend on the ability of Bcl-2 to target the kinase Raf-1 to mitochondrial membranes (*48*). The Bcl-2–dependent mitochondrial sequestration of Raf-1 could be a mechanism underlying our finding that, when Bcl-2 was preincubated in cytosol with one aliquot of mitochondria, it failed to protect a second portion of mitochondria added later (Fig. 4, C and D).

Our observations have identified the mitochondrial release of cytochrome c as a major target for the anti-apoptotic effects of Bcl-2. The ability of cytochrome c to activate CPP32-like proteases and cell death appears to be distinct from this protein's life-sustaining role in respiration. Cytochrome c is highly conserved in eukaryotes (*49*). If its function in apoptosis is also conserved, this would explain how Bcl-2 or similar molecules can effectively regulate most forms of apoptosis.

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Role for the Amino-Terminal Region of Human TBP in U6 snRNA Transcription

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Basal transcription from the human RNA polymerase III U6 promoter depends on a TATA box that recruits the TATA box–binding protein (TBP) and a proximal sequence element that recruits the small nuclear RNA (snRNA)–activating protein complex ($SNAP_c$). TBP consists of a conserved carboxyl-terminal domain that performs all known functions of the protein and a nonconserved amino-terminal region of unknown function. Here, the amino-terminal region is shown to down-regulate binding of TBP to the U6 TATA box, mediate cooperative binding with $SNAP_c$ to the U6 promoter, and enhance U6 transcription.

The TATA box–binding protein is a central transcription factor required for transcription by all three RNA polymerases. The highly conserved COOH-terminal domain performs all of the TBP functions examined so far, including binding to the TATA box and interacting with TBP-associated factors, general transcription factors, and activators (*1*). In vivo, this domain can functionally replace the full-length protein for all promoters tested in mammalian systems (*2*); and in yeast, strains carrying a TBP missing the NH_2-terminal domain are viable (*3*). The role of the nonconserved NH_2-terminal domain is unknown.

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Most RNA polymerase III promoters consist of gene-internal elements and recruit TBP as part of the TBP-containing transcription factor IIIB (TFIIIB), through protein-protein interactions with the DNA-binding TFIIIC (*4*). However, in some unusual cases, exemplified by the human U6 snRNA promoter, the promoter elements are located upstream of the transcription start site (*5*) and appear to recruit neither TFIIIC (*6*) nor the same TFIIIB complex recruited by RNA polymerase III promoters with gene-internal elements (*7*). Instead, the U6 promoter contains two basal promoter elements, a proximal sequence element (PSE), which recruits a multisubunit complex referred to as the SNAP complex ($SNAP_c$) or PSE transcription factor (*8*) and a TATA box,