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Pro-Apoptotic Activity of Transiently Expressed BCL-2 Occurs Independent of BAX and BAK

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Abstract BCL-2 suppresses apoptosis induced by a wide variety of stimuli in multiple cell types. Most of the in vitro studies that have examined the activity of BCL-2 have employed stable cell lines that ectopically express BCL-2. We have reported that BCL-2 is expressed at high levels in the absence of the 5′- and 3′-UTRs of the *Bcl-2* gene and transient high level of expression results in potent cell death (Uhlmann et al., [1998]: JBC 278:17926–17932). Expression of BCL-2 under the transcriptional control of the cognate 5′- and 3′-UTRs express lower levels of BCL-2 and does not cause cell death. Our present results suggest that in contrast to BCL-2, transient expression of BCL-xL does not induce cell death and coexpression of BCL-xL with the pro-apoptotic BCL-2 does not suppress cell death. The pro-apoptotic activity of BCL-2 appears to involve activation of the cytochrome c/caspase 9/caspase 3 pathway. Elevated levels of BCL-2 expression results in N-terminal cleavage of BCL-2 at a novel site different from a previously identified caspase cleavage site at Asp 34 by a non-caspase protease. Transient expression of a BCL-2 mutant lacking aa 51–85 within the loop region induces efficient cell death and N-terminal cleavage of BCL-2 while a different deletion mutant lacking aa 30–91 induces reduced levels of cell death in the absence of BCL-2 cleavage suggesting that N-terminal processing of BCL-2 may be an amplification event in BCL-2-mediated cell death. Overexpression of BCL-2 in a Bax-null human colon cancer cell line (HCT116Bax−/−) induces efficient cell death. The pro-apoptotic activity of BCL-2 is also observed in a Bax-null cells in which BAK expression is inhibited by stable RNAi expression. Our results suggest that BCL-2 contains an intrinsic pro-apoptotic activity and can induce apoptosis independent of BAX and BAK under specific conditions. J. Cell. Biochem. 89: 1102–1114, 2003.

Key words: BCL-2 family; apoptosis; untranslated region; BAX; BAK; RNAi

The Bcl-2 proto-oncogene was isolated from the t(14;18) chromosomal breakpoint [Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985]. The anti-apoptotic activity of BCL-2 was discovered in seminal studies where ectopic expression of Bcl-2 was shown to confer survival advantage to hematopoietic progenitor cells deprived of growth factors [Vaux et al., 1988; Hockenbery et al., 1990]. The in vivo anti-apoptotic activity of BCL-2 has been confirmed in mutant mouse models containing targeted mutations within the *Bcl-2* gene [Veis et al., 1993; Nakayama et al., 1994]. A number of cellular and viral homologs of BCL-2 (e.g., BCL-xL, BCL-w, MCL-1, EBV-BHRF1, and HHV8 vBCL-2) have also been shown to suppress apoptosis. The BCL-2 family proteins that exhibit anti-apoptotic activity are characterized by the presence of four BCL-2 homology (BH) domains (BH1-4). Among the various BCL-2 family anti-apoptosis proteins, BCL-2 and BCL-xL have been shown to be cleaved at the N-terminal region by caspases during apoptosis induced by treatment with anti-Fas antibody or by withdrawal of growth factors [Cheng et al., 1997; Clem et al., 1998]. The N-terminally processed forms of these proteins have been

Abbreviations used: UTR, untranslated region; BH, Bcl-2 homology; RNAi, RNA-mediated interference; Ad, adenovirus.

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postulated to function as pro-apoptotic molecules and amplify the apoptotic signaling. We have observed that transient over expression of BCL-2 results in a potent cell death activity [Uhlmann et al., 1998]. This cell death activity is manifested in the absence of any other apoptotic stimuli. Another group has reported that transient targeted mitochondrial expression of BCL-2 causes cell death while BCL-2 targeted to the ER promotes cell survival [Wang et al., 2001]. The *C. elegans* BCL-2 homolog, CED-9 has also been reported to promote cell death, in addition to cell survival [Hengartner and Horvitz, 1994]. Certain mutant backgrounds of *C. elegans* increase the cell death activity of CED-9. Thus, certain BCL-2 family anti-apoptosis proteins may promote cells death under specific conditions. Understanding the mechanisms that lead to functional reversal of these proteins could be beneficially exploited for therapeutic manipulation of their activity.

BCL-2 family proteins also contain proteins that are dedicated pro-apoptotic proteins. The pro-apoptotic members have been grouped into two classes. One class of proteins share more extensive homology with BCL-2 and contain BH1, 2, and 3 domains ('BH-123' proteins). The BH-123 proteins (also known as 'multi domain' proteins) include pro-apoptotic proteins BAX and BAK. The second class of pro-apoptotic proteins (known as 'BH3-only' proteins) shares only the BH3 domain with other BCL-2 family proteins. The expression of BH3-only proteins is activated in response to various apoptotic insults either by transcriptional or post-transcriptional mechanisms [reviewed in ref. Hunt and Evan, 2001]. In the canonical apoptosis paradigm of animal cells, various BH3-only proteins link different apoptotic stimuli to the core apoptosis machinery through the BH-123 proteins resulting in the release of apoptogenic factors from the mitochondria and in general mitochondrial dysfunction [reviewed by Reed and Green, 2002]. Studies with fibroblasts from mutant mice deficient for BAX and BAK have suggested that both proteins are essential for manifestation of apoptosis induced by diverse stimuli [Wei et al., 2001; Zong et al., 2001]. We have observed that a human colon cancer cell line deficient only for BAX is defective in manifestation of apoptosis induced by multiple stimuli [Theodorakis et al., 2002], suggesting that BAX plays a crucial role in driving cells to death in human epithelial cells. Here we report that the pro-apoptotic activity of BCL-2 does not require N-terminal processing and is manifested in human cells deficient for BAX and BAK.

MATERIALS AND METHODS

Cells and Viruses

Human A549 and 293 cells were grown in DMEM supplemented with 10% fetal bovine serum. Human colon carcinoma cell lines HCT116Bax (expresses one allele of the *Bax* gene) and HCT116BaxKO (null for the *Bax* gene) were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum. The adenoviral vectors Ad-LacZ (ΔE1), Ad-Bcl-2, and Ad-Bcl-2ΔUTR [Uhlmann et al., 1998] and Ad-Bik [Tong et al., 2001] were amplified and titrated on human 293 cells. All recombinant viruses lack the E1 region of the Ad5 genome and is replaced with cassettes that express the various proteins under the transcriptional control of the CMV promoter. Ad-Bcl-2 contains the 5′- and 3′-UTRs while Ad-Bcl-2ΔUTR lacks them. To construct Ad-Bcl-xLΔUTR and Ad-E1B-19K, the coding sequences of Bcl-xL and E1B-19K were cloned between *Hind*III and *Bam*HI sites of an Ad transfer vector, pLendCMV. The resultant plasmids were cotransfected with an Ad genomic plasmid pBHGE3 [Bett et al., 1994] into human 293 cells and overlaid with growth medium containing 2% fetal bovine serum and 1% noble agar. One week after transfection, plaques were isolated and screened for the expression of the recombinant proteins by immunoblot analysis. The recombinant viruses expressing Bcl-2 mutants, D34A (Asp residue at position 34 mutated to an Ala residue) and D31/34A (Asp residues at positions 31 and 34 mutated to Ala residues) [Cheng et al., 1997] were constructed in a similar fashion. Positive clones were further purified through a second round of plaque purification, amplified, and the progenies were titrated on 293 cells. BAK-depleted cell lines were generated by stable expression of RNAi. The oligonucleotides coding for the 21 nucleotide Bak RNAi were cloned into the vector pBS/U6 [Sui et al., 2002]. The Bax −/− cell line was transfected with the RNAi expressing vector along with pcDNA3 and 24 h after transfection, cells were selected with 600 μg of G418 for 2 weeks. Selected colonies were picked and established as cell lines and

analyzed for BAK protein expression by immunoblot analysis.

Antibodies and Immunoblot Analysis

The rabbit polyclonal antibody specific to BCL-2 was raised using the GST–BCL-2 fusion protein. An antibody specific for the C-terminus of BCL-2 was raised using a peptide corresponding to the transmembrane domain of BCL-2. Goat antibodies specific for the N-terminus of BCL-2 (SC 492G), actin (SC1615), and rabbit polyclonal antibodies to BCL-xL (SC1041), procaspase-3 (SC 7148), -8 (SC 7890), and -9 (SC 7885) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. A polyclonal BAK antibody (6–536) antibody was purchased from Upstate Biotechnology, Charlottesville, VA. Forty-eight hours after infection/transfection, both floating and adherent cells were collected and resuspended in 0.5 ml of sample lysis buffer. Fifty μl samples were analyzed by 15% SDS–PAGE. The separated proteins were electro transferred onto a nitrocellulose membrane and probed with a primary antibody followed by horseradish peroxidase conjugated secondary antibody (goat anti-rabbit and donkey anti-goat antibodies were purchased from Santa Cruz Biotechnology) and analyzed by chemiluminescent detection system (Amersham, Biosciences Corporation, Piscataway, NJ) according to the manufacturer specifications.

Cell Death Assay

A549 cells or HCT116 derived cells (1×10^6 cells/60 mm dish) were infected with various viruses at 100 PFU/cell. Forty-eight hours after infection, adherent cells were collected by trypsinization and combined with floating cells collected from the culture medium. The suspension was mixed with an equal volume of trypan blue and the cells that excluded the dye were counted. Cell death was also assessed by analysis of fragmented low molecular weight cellular DNA. For this analysis, cells (1×10^6) were infected with various viruses at 100 PFU/cell. Forty-eight hours after infection, both adherent and floating cells were collected, lysed, and the small molecular weight DNA was prepared by Hirt extraction [Subramanian et al., 1995], treated with RNAse and analyzed by electrophoresis on a 1.5% agarose gel. The cell viability in transfected cells were determined on the basis of the reporter gene expression in adherent cells. Human 293 cells (1×10^6 cells/35 mm dish) were transfected with 2 μg of pcDNA3 vector or BCL-2 expressing plasmids along with 1 μg of pRSVβ-gal reporter plasmid using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specification. Forty-eight hours after transfection, the adherent cells were collected and suspended in 200 μl of 250 mM Tris-Cl, pH 7.8. The cells were lysed by freeze–thaw and 50 μl samples of the supernatants were used to measure β-galactosidase activity by the ONPG method [Miller, 1972]. All experiments were repeated three times and the averages and standard deviations were calculated.

Cell Fractionation

A549 cells (1×10^7 cells/75 cm^2 flask) were infected with various viruses at 100 PFU/cell and 48 h after infection, mitochondria and S-100 fractions were prepared. Adherent cells were released into the growth medium by scraping with a cell scraper and were collected by centrifugation at 600 *g* for 10 min at 4°C. The cell pellets were washed twice with ice-cold PBS and resuspended in five volumes of lysis buffer (20 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 250 mM sucrose). The cells were dounced 50 times and the homogenates were centrifuged at 1,000 *g* for 10 min at 4°C to separate the nuclei and intact cells. The supernatants were centrifuged at 10,000 *g* for 20 min at 4°C and the resulting mitochondrial pellets were separated. The supernatants were centrifuged again at 100,000 *g* for 1 h at 4°C and the supernatant S-100 fractions were recovered.

RESULTS

Pro-Apoptotic Activity Is Unique to BCL-2

We have previously reported that expression of the Bcl-2 cDNA from an expression cassette that lacks the 5′- and 3′-UTRs of the *Bcl-2* gene results in elevated levels of mRNA and protein expression [Uhlmann et al., 1998]. Cells transfected with plasmid constructs or infected with an Ad vector that expresses the BCL-2 ORF (in the absence of 5′- and 3′-UTR) undergo profound cell death. To determine if such activity could be observed with other anti-apoptosis proteins, we constructed an Ad vector that expresses Bcl-xL and examined its effect on cell death. Human A549 cells were infected with adenovirus vectors that express *E. coli* lac Z, Bcl-2 (+UTR), or

Bcl-2 (ΔUTR), Bcl-xL(ΔUTR), or BIK (BH3-only pro-apoptotic member) and the effect on cell death was determined by analysis of intracellular low molecular weight DNA (Fig. 1A) and by quantification of cell viability (Fig. 1B). Cells infected with Ad-Bcl-2ΔUTR contained significant amount of fragmented low molecular weight DNA. The level of DNA fragmentation was comparable to that seen in cells infected with the vector that expresses the BH3-only pro-apoptotic protein BIK (Ad-Bik). In contrast, cells infected with Ad-Bcl-2 (+UTR) or Ad-Bcl-xL (ΔUTR) did not contain fragmented DNA. Consistent with the DNA fragmentation, there was significant loss of viability of cells infected Ad-Bcl-2 (ΔUTR) compared to cells infected with Ad-Bcl-2 or Ad-Bcl-xL (ΔUTR) (Fig. 1B). Infection of cells with Ad-Bcl-xL even under higher multiplicities of infection (e.g., 200 PFU/cell) did not induce any significant cell death (not shown) in spite of high levels of protein expression (Fig. 1C). These results suggest that the pro-apoptotic activity induced by BCL-2 is not manifested by BCL-xL.

We then determined if the pro-apoptotic activity of BCL-2 could be suppressed by BCL-xL. For this purpose, A549 cells were coinfected with Ad-Bcl-2 (ΔUTR) and Ad-Bcl-xL (ΔUTR) and cell viability was determined (Fig. 2). Coinfection of Ad-Bcl-xL (ΔUTR) at two different multiplicities (50 PFU or 100 PFU/cell) did not increase cell viability compared to cells infected with Ad-Bcl-2 (ΔUTR) alone (50 PFU/cell). In similar coinfection experiments, we have observed that BCL-xL could readily suppress BIK-mediated cell death (not shown). These results suggest that the pro-apoptotic activity of BCL-2 may not be suppressed by BCL-xL.

Effect of Loop Mutants on Cell Death and N-Terminal Cleavage

A previous study has suggested that BCL-2 is converted into a pro-apoptotic molecule by cleavage at a caspase site at Asp 34 in cells exposed to apoptotic stimuli such as treatment with the Fas antibody or withdrawal of growth factors [Cheng et al., 1997]. Therefore, we examined if cleavage of BCL-2 can also occur under conditions of overexpression. A549 cells were infected with Ad-Bcl-2 (ΔUTR) and analyzed by immunoblot analysis using two different polyclonal antibodies specific to BCL-2. When the blots were probed with the anti-

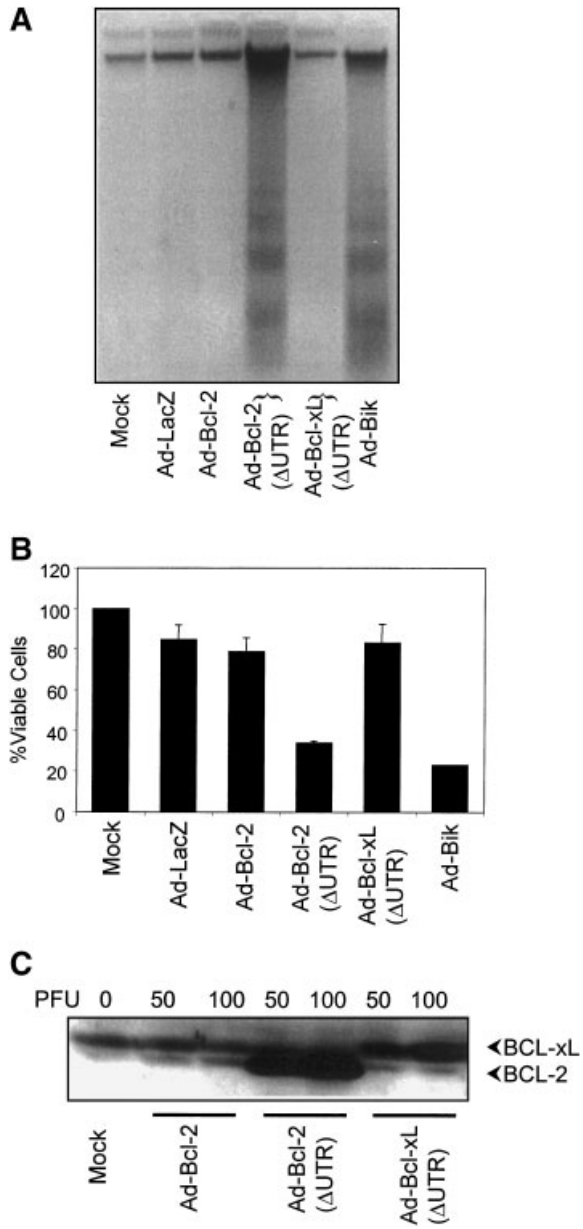

Fig. 1. Effect of transient expression of BCL-2 and BCL-xL on cell death. Human A549 cells were infected with 100 PFU/cell of Ad vectors expressing BCL-2 or BCL-xL and the effects on cell death were determined by analysis of intracellular DNA (**A**) or by measuring cell viability by trypan blue staining (**B**). An immunoblot showing the levels of protein expression under different multiplicities of infection is shown in (**C**). The protein blot was probed with a mixture of polyclonal antibodies specific to BCL-2 and BCL-xL. The vector Ad-Bcl-2 contains the expression cassette that includes the Bcl-2 UTR regions while Ad-Bcl-2 ΔUTR and Ad-Bcl-xL (ΔUTR) lack the respective UTR regions.

body directed against the whole BCL-2 protein (BCL-2 Ab), in addition to the 26 kDa band, a 20 kDa band (tBCL-2) was consistently observed (Fig. 3A). This band was not observed when

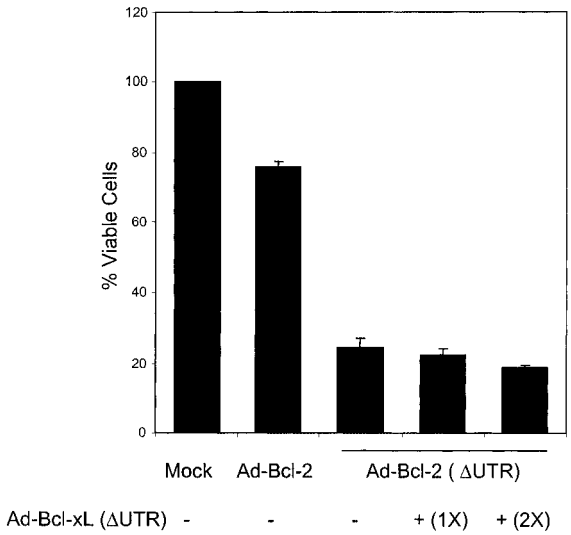

Fig. 2. Effect of BCL-xL on BCL-2-mediated cell death. Human A549 cells were infected singly with Ad-Bcl-2 or Ad-Bcl-2 (ΔUTR) at 50 PFU/cell or coinfected with Ad-Bcl-xL at 50 (indicated as 1×) or 100 PFU/cell (indicated as 2×). The cell viability was determined by trypan blue staining.

the blots were probed with a polyclonal antibody specific for the N-terminus (N-19 Ab). The tBCL-2 band was not observed in blots containing proteins from cells expressing Ad-Bcl-2 (+UTR) even when higher concentrations of the protein extracts (adjusting to comparable levels of BCL-2) were analyzed. To determine if tBCL-2 is generated by cleavage of BCL-2 at a previously characterized caspase cleavage site at D34 [Cheng et al., 1997], cells were infected with Ad vectors expressing two mutants (D34A, D31/34A) that obliterate the caspase cleavage site D34 [Cheng et al., 1997]. Cells infected with these mutants also contained the 20 kDa (tBCL-2) band (Fig. 3A). Importantly, expression of these mutants also induced significant cell death (Fig. 3B). These results suggest that the proteolytic cleavage site for tBCL-2 observed under conditions of overexpression may be distinct from the previously reported caspase site at D34 and caspase cleavage at D34 may not significantly contribute to the pro-apoptotic activity observed during transient expression.

Human and animal BCL-2 proteins contain an additional Glu residue (D10) near the N-terminus. To determine if D10 could be a potential target site for caspase mediated cleavage of BCL-2, we introduced a mutation at D10 (D10A). This mutation was also introduced under the background of the D31/34A mutation

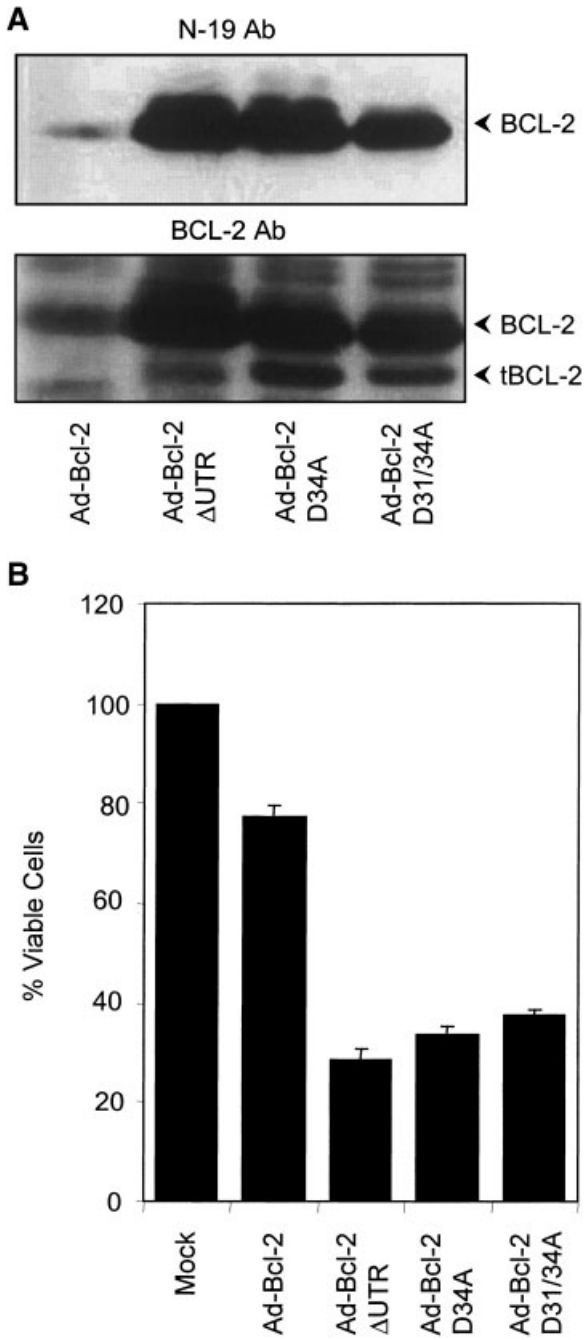

Fig. 3. Effect of caspase cleavage site mutants on cell death and protein processing. Human A549 cells were infected with Ad vectors expressing wt Bcl-2 (±UTR) or mutants that affect the caspase cleavage site at Asp 34. **A**: The expression of BCL-2 protein was analyzed using an antibody raised against an N-terminal peptide (N-19 Ab) or against the whole protein (BCL-2 Ab). The N-terminally processed form of BCL-2 is indicated as tBCL-2. **B**: Cell viability was determined by trypan blue staining.

(i.e., all Asp residues within the loop region were changed to Ala residues). The effect of these mutants on the cell death was determined by transient transfection. Human 293 cells were transfected with a plasmid expressing the *E. coli lac Z* gene with plasmids expressing BCL-2 (+UTR) or BCL-2 (ΔUTR) or various BCL-2 mutants (in ΔUTR background). Forty-eight hours after transfection, the β-galactosidase activity in surviving adherent cells was determined (Fig. 4C). Mutant D10A induced significant loss in β-galactosidase like the parental construct Bcl-2 (ΔUTR). We also determined the effect of two deletion mutants (in ΔUTR background) within the loop region lacking aa 51–85 [Uhlmann et al., 1996] or aa 30–91 [Hunter et al., 1996]. The mutant Δ51–85 also induced severe reduction in β-galactosidase activity while the mutant Δ30–91 induced only partial loss in cell viability (compared to cells transfected with the vector or Bcl-2 (+UTR).

The relative levels of BCL-2 protein expression in 293 cells transfected with various mutants were determined by immunoblot analysis (Fig. 4A,B). For this purpose, the transfected cells were kept in media containing the broad-spectrum caspase inhibitor zVAD-fmk. This treatment was deemed necessary since preliminary experiments suggested that estimation of the levels of BCL-2 in transfected cells was unreliable due to rapid cell death in cells transfected with pro-apoptotic Bcl-2. Cells transfected with mutants D10A, D10/31/34A expressed elevated levels of BCL-2 (probed with BCL-2 Ab) compared to cells transfected with Bcl-2 (+UTR). Interestingly, cells transfected with Bcl-2 (ΔUTR) or mutant D10A or D10/31/34A contained significant amount of tBCL-2. The protein expression in cells transfected with deletion mutants Δ51–85 and Δ30–91 was analyzed using a polyclonal antibody raised against the C-terminal region of BCL-2 (Fig. 4B). Cells transfected with Δ51–85 contained significant levels of tBCL-2 while cells transfected with Δ30–91 did not contain detectable tBCL-2. This issue was confirmed in analyses employing SDS–PAGE with concentrations of acrylamide and immnunoblot analyses. These results suggest that tBCL-2 may be generated by a non-caspase protease by cleaving BCL-2 within the loop region. Since mutant Δ30–91 (defective in tBCL-2 formation) is significantly reduced in cell death activity, our results suggest that N-terminal processing of BCL-2 may be important for full manifestation of the apoptotic activity. However, such protein processing may not be fully essential for the onset of cell death.

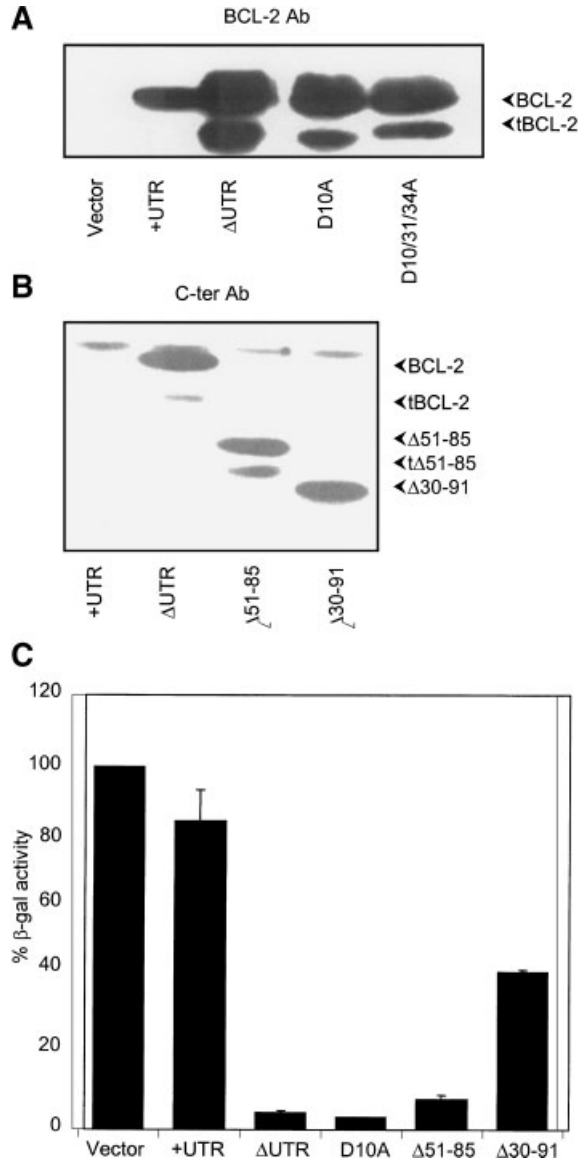

Fig. 4. Effect of BCL-2 loop mutants on protein processing and cell viability. Human 293 cells were transfected with the reporter plasmid (pRSV-β gal) along with the vector (pcDNA3) or plasmids expressing wt BCL-2 (±UTR) or various mutants (ΔUTR). The transfected cells were maintained in the presence of 50 μM zVAD-fmk for the analysis of BCL-2 protein (analyzed by immuno blots in **A** and **B**) or in the absence for the measurement of cell viability (**C**). The cell viability was measured on the basis of β-galactosidase activity in adherent cells. Arrowheads: BCL-2 indicates full-length BCL-2; tBCL-2 indicates truncated BCL-2. The BCL-2 sequences deleted in the mutant proteins (Δ51–85 and Δ30–91) are also indicated.

Caspase Activation

We examined if BCL-2 manifests its pro-apoptotic activity through the canonical mitochondrial injury pathway. A549 cells were infected with recombinant adenoviruses that express various BCL-2 family proteins and the S100 fractions were examined for the levels of cytosolic cytochrome c and the levels of various procaspases (4). Cells infected with Ad-Bcl-2 (ΔUTR) as well as cells infected with Ad-Bik contained significant levels of cytosolic cytochrome c (Fig. 5). In contrast, cells expressing Bcl-2 (+UTR) or Bcl-xL or E1B-19K or LacZ did not contain detectable levels of cytosolic cytochrome c. Consistent with the results on cytochrome c, cells expressing the pro-apoptotic version of BCL-2 or BIK contained reduced levels of procaspase-9 and procaspase-3. There was no significant difference in the levels of procaspase-8 among the cells expressing various BCL-2 family proteins. These results suggest that transient overexpression of BCL-2 may result in apoptosis by causing mitochondrial injury.

BAX Independent Cell Death

BAX plays a central role in mediating apoptosis in animal cells. The relative levels of BAX and BCL-2 have been suggested to dictate the death vs. survival decisions [Oltvai et al., 1993]. Recent studies with fibroblast cells from mutant mice suggest that BAX and BAK are crucial for apoptosis induced by multiple stimuli [Wei et al., 2001; Zong et al., 2001]. We have shown that a human epithelial cancer cell line (HCT116 BaxKO) deficient for Bax is defective in manifestation of apoptosis by different stimuli such as ligands for death receptors, DNA damaging agents, and ectopic expression of the BH3-only protein BIK [Theodorakis et al., 2002]. To determine if the pro-apoptotic activity of BCL-2 is dependent on BAX, we employed two matched colon cancer cell lines that are isogenic except for Bax [Zhang et al., 2000]. The cell line HCT116Bax is heterozygous for the Bax alleles (Bax+/−) and expresses significant levels of BAX protein. In the cell line, HCT116BaxKO the second Bax allele has been knocked out by homologous recombination (Bax−/−). These cell lines were infected with Ad vectors that express various BCL-2 family proteins and their apoptotic activity was determined by microscopic examination (Fig. 6A) and by analysis of fragmented cellular DNA (Fig. 6B). In Bax-expressing (Bax+/−) cells, the BH3-only pro-apoptotic protein BIK as well as BCL-2 (ΔUTR) induced significant cell death as evidenced by the round apoptotic cells in Figure 6A and by the presence of fragmented DNA in the infected cells as seen in Figure 6B. In Bax-null (Bax−/−) cells, BIK did not induce any detectable cell death while BCL-2 (ΔUTR) induced significant cell death. Infection with Ad vectors that express other BCL-2 family proteins, BCL-xL or E1B-19K did not induce detectable cell death

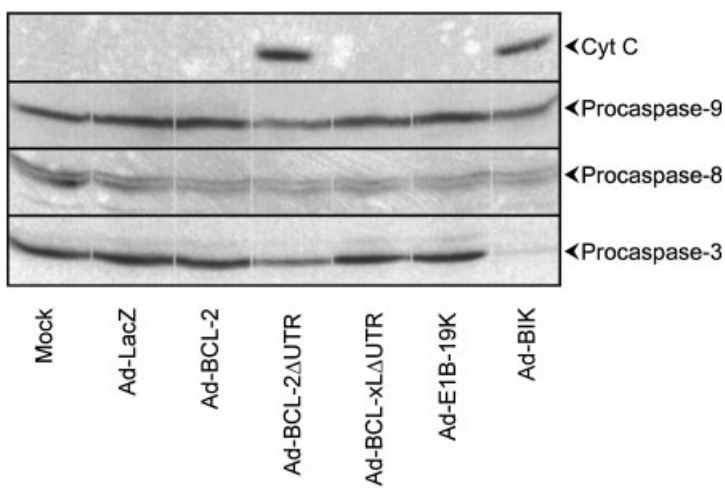

Fig. 5. Effect of BCL-2 on cytochrome c release and caspase activation. A549 cells were infected with various Ad expression vectors and 48 h after infection, the cell lysates were analyzed for procaspase-9, procaspase-8, and procaspase-3 and the S-100 fractions were analyzed for cytochrome c by immunoblot analysis. In the image of the blot, white lines were inserted between lanes for better visualization of individual lanes.

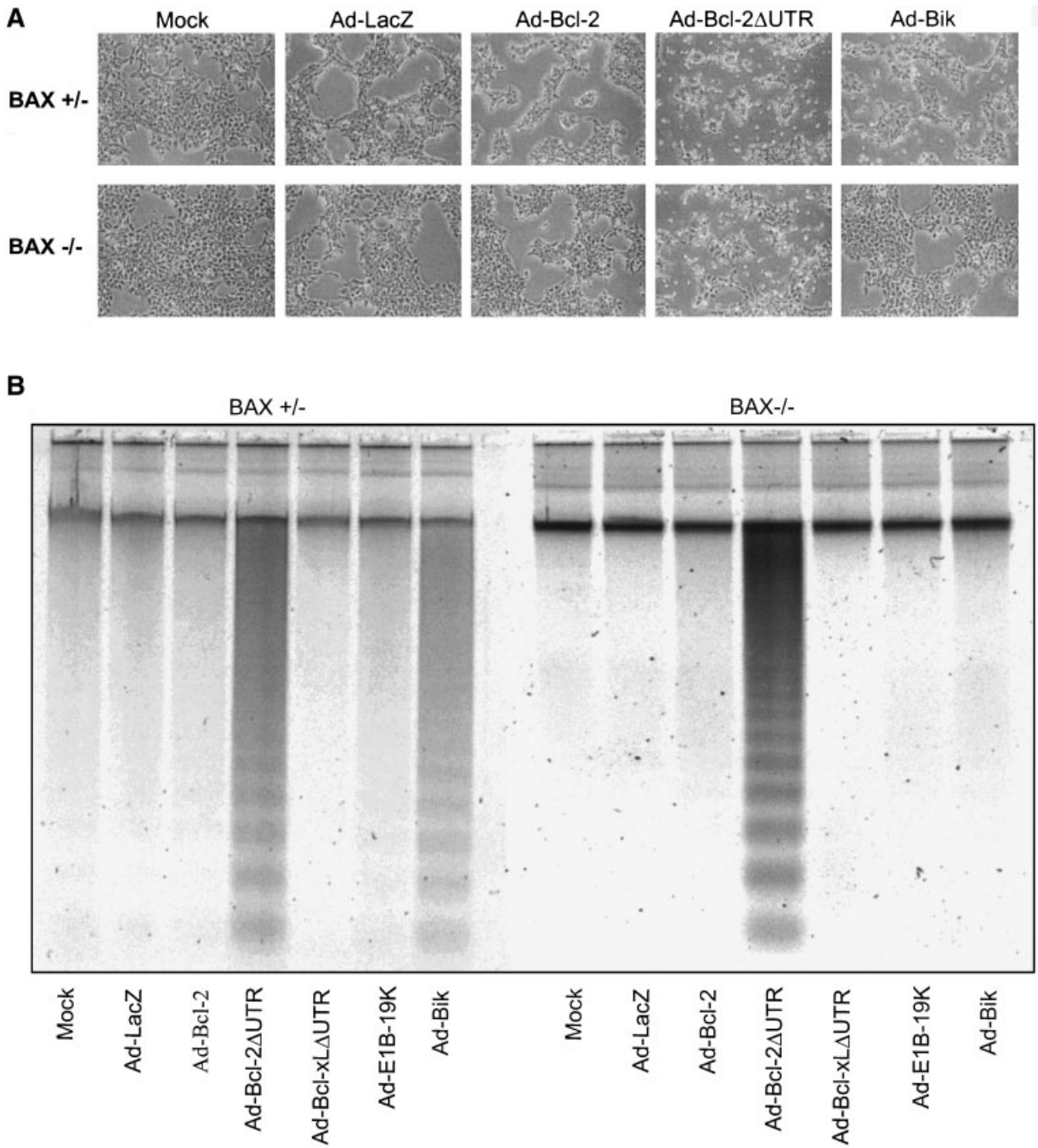

Fig. 6. Effect of BCL-2 expression in Bax-proficient and deficient colon cancer cells. Human colon cancer cell line HCT116Bax (+/−) and Bax (−/−) cells were infected with various Ad vectors and the effect on cell death was determined by photomicroscopic examination (**A**) and analysis of intracellular low molecular weight DNA (**B**).

(Fig. 6B). These results suggest that BCL-2 can induce cell death in the absence of BAX (while BIK requires BAX).

BAK-Independent Cell Death

Studies with mouse embryo fibroblasts have suggested that BAX and BAK are functionally redundant. A number of diverse apoptotic stimuli have been shown to signal either through BAX or BAK and the cells deficient for both are resistant to apoptosis [Wei et al., 2001; Zong et al., 2001]. To determine if BCL-2 can induce cell death in the absence of both BAX and BAK, we established BAK-depleted cell lines from the

Bax-null HCT116BaxKO cells by stable transfection of a DNA vector [Sui et al., 2002] that expresses Bak-specific RNAi under the transcriptional control of the U6 promoter. Two different clonal cell lines that express greatly reduced levels of BAK (Fig. 7A) were infected with Ad-LacZ or Ad-Bcl-2 or Ad-Bcl-2ΔUTR and the effect on cell death was determined by microscopic examination (Fig. 7B), cell viability by trypan blue staining (Fig. 7C), and by analysis of fragmented cellular DNA (Fig. 7D). In both BAK-depleted cell lines (BAK-RNAi-1 and BAK-RNAi-2) Ad-Bcl-2 (ΔUTR) induced significant cell death. These results suggest that BCL-2 can induce cell death in cells deficient for both BAX and BAK. Although, the effect of residual BAK (<10%) present in cell lines can not be ruled out, the observation that both BAK-depleted cell lines (varying in the levels of residual BAK expression) induce similar levels of cell death compared to cells that express wt levels of BAK (Fig. 7C) suggest that BCL-2 may cause cell death independent of BAK. Taken together, it appears that both BAX and BAK are not required for manifestation of the pro-apoptotic activity of BCL-2.

DISCUSSION

We have expanded on our original observation that transient overexpression of BCL-2 causes potent cell death in the absence of any external stimuli [Uhlmann et al., 1998]. Although BCL-2 is largely an anti-apoptotic protein (under conditions of stable expression), our results have suggested that BCL-2 has an intrinsic pro-apoptotic activity. It is possible that the pro-apoptotic activity may serve an internal regulatory role to modulate the activity of BCL-2. This notion is supported by genetic analysis of the activity of the *C. elegans* gene, *Ced-9*. The CED-9 protein appears to exist in two distinct forms that either promotes cell survival or cell death [Hengartner and Horvitz, 1994]. Other BCL-2 family members have also been reported to exhibit opposing activities depending on the context. The BH123 protein BAX functions as a pro-apoptotic protein under a wide array of settings. However, it has also been reported to inhibit apoptosis in neuronal cells [Lewis et al., 1999]. Similarly, the pro-apoptotic protein BAK has been reported to inhibit apoptosis in an EBV-transformed B-cell line [Kiefer et al., 1995]. In contrast to BCL-2, BCL-xL does not appear to exhibit the pro-apoptotic activity. This may explain the widely observed phenomenon that BCL-xL is a more potent anti-apoptotic protein than BCL-2.

The pro-apoptotic activity of BCL-2 may have physiological relevance. The Bcl-2 proto-oncogene has unusually long 5′- and 3′-UTRs. The 3′-UTR has been reported to contain AU-rich mRNA destabilizing elements that are implicated in down regulation of the *Bcl-2* gene expression [Schiavone et al., 2000; Lapucci et al., 2002]. Similarly, the 5′-UTR contains cis-acting translation inhibitory sequence [Harigai et al., 1996]. These structural features may have evolved for down regulation of BCL-2 and to regulate the levels of BCL-2 expression under different contexts during development. Many cancers have genetic alterations (e.g., t(14;18) chromosomal translocation in human B lymphomas) that result in activation of BCL-2 expression. It is possible that such tumor cells might have acquired resistance to BCL-2 toxicity during tumor expansion. We have observed that the BCL-2 loop mutant (Δ51–85) that induces potent cell death in transient assays (Fig. 4) confers a strong anti-apoptotic property to cells that have been selected for stable BCL-2 expression [Uhlmann et al., 1996]. It is also possible that high levels of BCL-2 may be tolerated in tumor cells and in normal cells that are exposed to chronic or acute apoptotic stimuli. For example, studies with transgenic mouse models suggest that overexpression of BCL-2 causes apoptosis in normal photoreceptors while reducing apoptosis in photoreceptors of animals that express a mutant form of rhodopsin [Chen et al., 1996].

The mechanisms by which the opposing activities of BCL-2 are mediated remain to be elucidated. We have observed an N-terminally truncated version of BCL-2 (tBCL-2) is generated by a non-caspase protease (not inhibited by zVAD-fmk) during transient overexpression. It is possible that tBCL-2 may function like the BH123 (multi domain) pro-apoptotic proteins such as BAX and BAK and contribute to cell death to some extent. Our observation that the deletion mutant Δ30–91 is defective in formation of tBCL-2 and causes reduced levels of cell death suggesting that formation of tBCL-2 may be one of the contributing factors for BCL-2-mediated cell death. However, our results cannot distinguish if generation of tBCL-2 is an

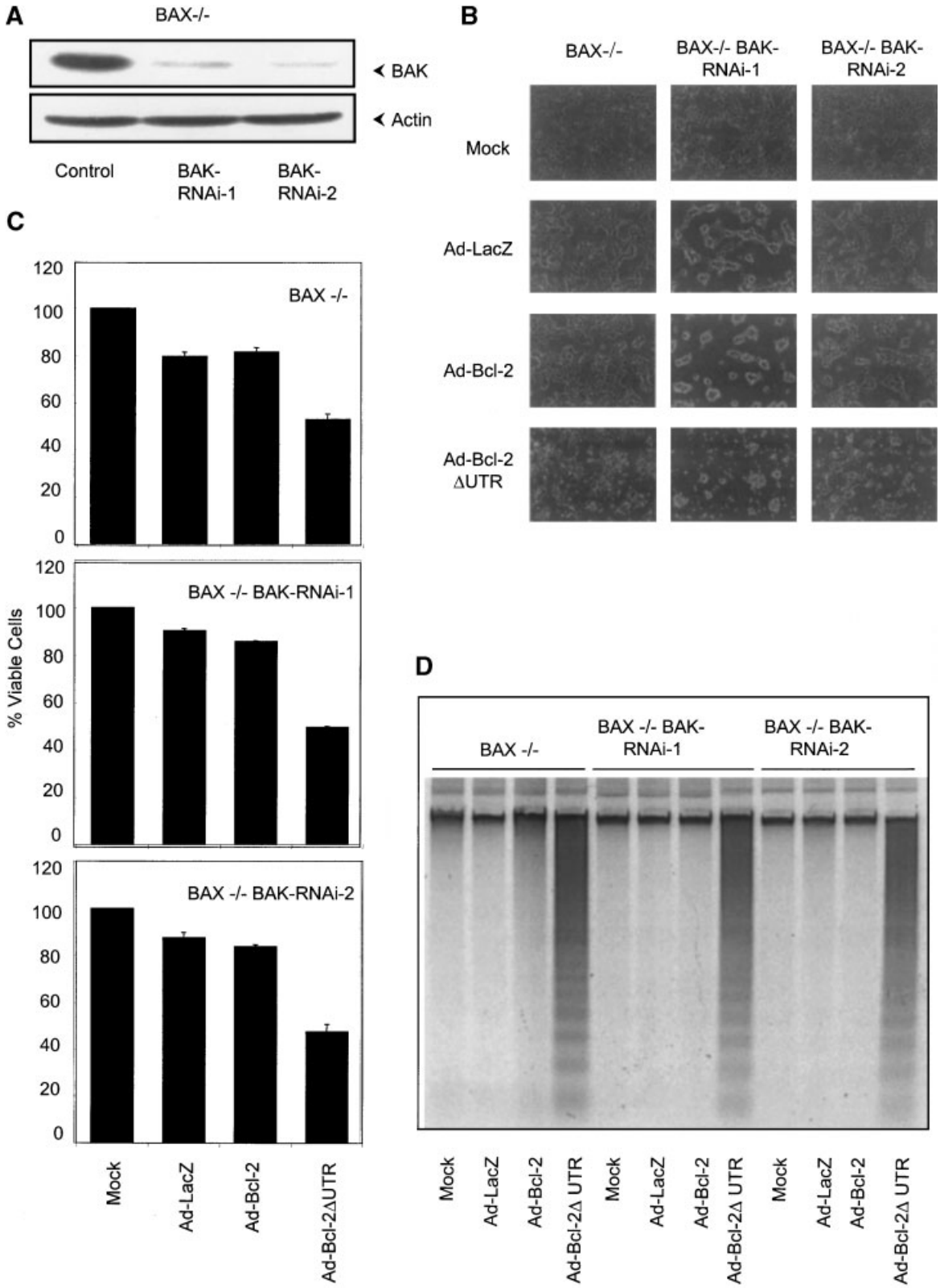

Fig. 7. Effect of BCL-2 expression in Bax and Bak-deficient colon cancer cells. Bak-deficient cell lines (BAK-RNAi-1 and BAK-RNAi-2) were established by stable expression of RNAi. The expression of BAK and actin proteins was analyzed by immunoblot analysis of the parental cell line (control) and the BAK-depleted cell lines (**A**). The effects on cell death are shown in **B**–**D**. The cell viability was determined by photomicroscopic examination (B), trypan blue staining (C), and by analysis of intracellular DNA (D).

early or late event. Since mutant Δ30–91 appears to induce a reduced levels of cell death (in the absence of BCL-2 cleavage) compared to vector or Bcl-2 (+UTR) transfected cells (Fig. 4C), it is possible that tBCL-2 formation may be a late amplification event. We have shown that tBCL-2 is generated by a non-caspase protease (Fig. 4A,B). The apoptogenic factors released from mitochondria during late stages of apoptosis include a serine protease Omi/HtrA2 [Suzuki et al., 2001; Hegde et al., 2002; Martins et al., 2002; Verhagen et al., 2002]. It is possible that BCL-2 may be a target for such proteases. BCL-2 has been reported to initiate a caspase activation program independent of the apoptosome [Marsden et al., 2002]. It remains to be determined if such a caspase activation program is also activated during initial stages of apoptosis induced by transient BCL-2 expression.

We have shown that BCL-2 efficiently induces cell death in the absence of BAX. Additionally, BCL-2 also induces efficient cell death in Bax-null cells that have been depleted for BAK. Our results suggest that BCL-2 mediates its pro-apoptotic activity independent of both BH123 pro-apoptotic proteins. This is in contrast to most other external and internal apoptotic stimuli that have obligatory requirement for the BH123 proteins [Wei et al., 2001; Zong et al., 2001]. Thus, BCL-2 appears to induce cell death by acting like BAX and BAK during transient expression. We have observed that the late events (cytochrome c/caspase-9/caspase-3 activation) in apoptosis induced by BCL-2 may be similar to that observed in BAX/BAK-mediated apoptosis. However, while BAX/BAK-mediated apoptosis could be efficiently suppressed by BCL-xL, BCL-2-induced cell death is not suppressed by BCL-xL.

The solution structures of BCL-2 [Petros et al., 2001] and BCL-xL [Muchmore et al., 1996] are remarkably similar to the structure of BAX [Suzuki et al., 2000]. The structures of BCL-xL and BCL-2 have revealed the N-terminal α-helical region (α-1) forms extensive hydrophobic interactions with the hydrophobic groove on the surface of the protein formed by BH1 (α-5), 2 (α-6), and 3 (α-2) regions. A similar mode of domain interactions have also been observed in BAX [Suzuki et al., 2000]. The structural studies have revealed that BCL-2 and BCL-xL exist as monomers in solution. It is possible that under higher levels of protein expression, BCL-2 may undergo "forced" oligomerization that may lead to apoptosis. Further, conformational changes in BCL-2 or removal of α-1 by proteolytic processing may reduce interaction with hydrophobic groove leading to homo-oligomerization of BCL-2. The activities of anti-apoptotic proteins, BCL-2 and BCL-xL have also been reported to be modulated by protein modifications. For example, phosphorylation of BCL-2 [Haldar et al., 1996, 1998] and deamidation of BCL-xL [Deverman et al., 2002] have also been reported to down regulate the anti-apoptosis activity of these proteins. It remains to be determined if any protein modifications may be involved in pro-apoptotic conversion of BCL-2. Irrespective of the exact mechanism, it appears that the opposing activities of BCL-2 can be exploited for therapeutic modulation of BCL-2 activities.

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