Bax Can Antagonize Bcl-X\textsubscript{L} during Etoposide and Cisplatin-induced Cell Death Independently of Its Heterodimerization with Bcl-X\textsubscript{L}\textsuperscript{*}

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Bax, a member of the Bcl-2 family of proteins, has been shown to promote apoptosis while other members of the family, including Bcl-X\textsubscript{L} and Bcl-2, inhibit cell death induced by a variety of stimuli. The mechanism by which Bax promotes cell death is poorly understood. In the present report, we assessed the ability of Bax to antagonize the death repressor activity of Bcl-X\textsubscript{L} during chemotherapy-induced apoptosis in the lymphoid cell line, FLS.12. Expression of wild-type Bax counteracted the repressor activity of Bcl-X\textsubscript{L} against cell death mediated by VP-16 and cisplatin. We performed site-directed mutagenesis of the BH1, BH2, and BH3 homology regions in Bax to determine the ability of wild-type and mutant Bax to heterodimerize with Bcl-X\textsubscript{L} and to antagonize the protective effect of Bcl-X\textsubscript{L} against chemotherapy-induced apoptosis. Bax proteins expressing alanine substitutions of the highly conserved amino acids glycine 108 in BH1, tryptophan 151 and 158 in BH2, and glycine 67 and aspartic acid 68 in BH3 retained their ability to promote chemotherapy-induced cell death that was inhibited by Bcl-X\textsubscript{L} and to form heterodimers with Bcl-X\textsubscript{L}. Bax proteins containing deletions of the most highly conserved amino acids in BH1 (Δ102–112) and BH2 (Δ151–159) maintained the ability of Bax to antagonize the death repressor activity of Bcl-X\textsubscript{L} and to associate with Bcl-X\textsubscript{L}. However, Bax with BH3 deleted did not form heterodimers with Bcl-X\textsubscript{L} but retained its ability to counter the death repressor activity of Bcl-X\textsubscript{L}. These results demonstrate that the conserved BH3, but not BH1 or BH2, homology region of Bax is necessary for its interaction with Bcl-X\textsubscript{L} in mammalian cells. Furthermore, our results indicate that Bax does not require BH1, BH2, BH3, or heterodimerization with Bcl-X\textsubscript{L} to counter the death repressor activity of Bcl-X\textsubscript{L}. Therefore, Bax can antagonize Bcl-X\textsubscript{L} during VP-16 and, in a lesser degree, during cisplatin-induced cell death independent of its heterodimerization with Bcl-X\textsubscript{L}.

Apoptosis, a morphologically distinguished form of programmed cell death, is critical not only during development and tissue homeostasis but also in the pathogenesis of a variety of diseases including cancer, autoimmune disease, viral infection, and neurodegenerative disorders (1–6). Moreover, many chemotherapy drugs used to treat cancers are thought to destroy tumor cells through activation of apoptosis (7, 8). The precise mechanisms that control apoptosis have not been elucidated; however, it appears that this form of cell death is regulated by a genetic program involving both effectors andappers (6). The bcl-2 gene, the first member of a rapidly expanding family of genes that regulate apoptosis, was initially isolated from the t(14:18) chromosomal translocation found in human B-cell follicular lymphomas and was subsequently shown to repress cell death triggered by a diverse array of stimuli (9–11). The Bcl-2 family of proteins share conserved regions termed Bcl-2 homology domain 1, 2, and 3 (BH1, BH2, and BH3) (12, 13). Two other family members, Bax and Bcl-X\textsubscript{L}, are also known to regulate apoptosis. Bcl-X\textsubscript{L} represses cell death (14), while Bax counters the death repressor activity of Bcl-2 and accelerates cell death induced by growth factor withdrawal (15). The biochemical mechanism by which Bcl-2 and Bcl-X\textsubscript{L} repress cell death is unknown. However, certain highly conserved amino acids within BH1 and BH2 are required for Bcl-2 to repress cell death and associate with Bax as shown by mutation analysis (12). Although Bcl-2 repression of cell death is postulated to occur through heterodimerization with Bcl-X\textsubscript{L} (12), mutations in Bcl-X\textsubscript{L} of these conserved regions showed that Bcl-X\textsubscript{L} can protect against apoptosis independently of Bax (16). Thus, mutations which disrupted the association between Bcl-X\textsubscript{L} and Bax, still preserved 70–80% of the wild-type anti-apoptotic activity of Bcl-X\textsubscript{L}, suggesting that Bcl-X\textsubscript{L} can protect against apoptosis independent of Bax (16).

Bak, another member of the Bcl-2 family of proteins, has been shown to both accelerate and inhibit apoptosis (17–19). In FL5.12 cells deprived of interleukin 3 (IL-3),\textsuperscript{1} exogenous Bax expression promoted cell death and countered the anti-apoptotic action of Bcl-2 (17). Conversely, Bak overexpression inhibited cell death in the lymphoblastoid cell line, WI-L2, upon serum withdrawal and treatment with the cytotoxic agent menadione, in contrast to Bax that had no effect in this cell line (19). Thus, Bak and Bax are able to accelerate cell death, but Bak unlike Bax can also inhibit cell death (17), which suggests that they can act through different mechanisms. Structure and function analysis of Bak revealed that to interact with Bcl-X\textsubscript{L}, Bak required a region termed BH3 that is also conserved in Bax, Bcl-X\textsubscript{L}, and Bcl-2 (13). Although Bax has been shown to counter Bcl-2 during apoptosis triggered by IL-3 withdrawal (15), its role in modulating Bcl-X\textsubscript{L} function is unclear. Furthermore, the function of the conserved homology regions of Bax have not

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\textsuperscript{1} The abbreviations used are: IL-3, interleukin 3; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; wt, wild type; mAb, monoclonal antibody.
been defined. In this report, we assessed the ability of Bax to modify the death repressor activity of Bcl-X\textsubscript{L} during chemotherapy-induced apoptosis. In addition, we performed site-directed mutational analysis of the conserved BH1, BH2, and BH3 homology regions of Bax to determine their functional requirement for Bcl regulation of Bcl-X\textsubscript{L} activity in mamma-

**EXPERIMENTAL PROCEDURES**

**Generation of bax and bcl-x\textsubscript{L} Constructs**—An influenza virus hemagglutinin (HA) epitope was introduced at the amino terminus of Bax by PCR amplification using a murine bax cDNA template previously described (15). The Flag epitope-tagged Bcl-X\textsubscript{L} has been described elsewhere (20). Flag-tagged bcl-x\textsubscript{L} inserts were cloned into the EcoRI site of pSFFV-Neo expression vector (15).

**Western Blot and Immunoprecipitation Analysis**—The expression of Flag-Bcl-X\textsubscript{L} was randomly selected and transfected simultaneously with Flag-Bcl-X\textsubscript{L} and HA-Bax constructs in the parental FL5.12.Bcl-X\textsubscript{L} cells following wt and mutant HA-Bax expression compared to wt HA-Bax clone 102–112 (Fig. 2A). The expression of Flag-Bcl-X\textsubscript{L} in the derived cell clones remained similar to that of the parental FL5.12.Bcl-X\textsubscript{L} cells following wt and mutant HA-Bax transfection, selection in hygromycin, and expression of exog-

**RESULTS**

**Bax Does Not Require the Most Conserved Amino Acids in B1H to Antagonize the Death Repressor Activity of Bcl-X\textsubscript{L}**—To determine if Bax requires BH1 to counter the death repressor activity of Bcl-X\textsubscript{L} during chemotherapy-induced cell death, we generated mutations of the most conserved amino acids in BH1 by changing glycine 106 (G106) to an alanine and deleting residues 102 to 112 (Δ102–112) (Fig. 1B). These amino acids in BH1 were required for Bcl-2 and Bcl-X\textsubscript{L} to form heterodimers with Bax and for their anti-apoptotic activity against IL-3 withdrawal (12, 16). Constructs containing HA-bax wt, HA-bax G106S, and HA-bax Δ102–112 were transfected into the parental FL5.12.Flag-Bcl-X\textsubscript{L} cell line at each day after the addition of drug. Statistical analysis was performed using STATISTICA software (Stats Soft, Tulsa, OK).

**FIG. 1. Schematic representation with amino acid sequence comparison of BH1, BH2, and BH3 conserved regions of Bax and Bcl-X\textsubscript{L}**—A, identical amino acids in each region are in bold type. Numbers indicate position of residues in Bax. B–D, representation of alanine substitutions or deletion mutations generated for BH1, BH2, and BH3 conserved regions of Bax, respectively. Dashes represent no residue change while, an A represents an alanine substitution and stars represent deleted amino acids from wild type Bax.

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**Experimental Procedures**

**Generation of bax and bcl-x\textsubscript{L} Constructs**—An influenza virus hemagglutinin (HA) epitope was introduced at the amino terminus of Bax by PCR amplification using a murine bax cDNA template previously described (15). The Flag epitope-tagged Bcl-X\textsubscript{L} has been described elsewhere (20). Flag-tagged bcl-x\textsubscript{L} inserts were cloned into the EcoRI site of pSFFV-Neo expression vector (15). Proper orientation was determined by restriction mapping and the authenticity of the inserts was confirmed by DNA sequencing. The specific activity of the inserts was confirmed by DNA sequencing.

**Western Blot and Immunoprecipitation Analysis**—The expression of Flag-Bcl-X\textsubscript{L} was randomly selected and transfected simultaneously with Flag-Bcl-X\textsubscript{L} and HA-Bax constructs in the parental FL5.12.Bcl-X\textsubscript{L} cells following wt and mutant HA-Bax expression compared to wt HA-Bax clone 102–112 (Fig. 2A). The expression of Flag-Bcl-X\textsubscript{L} in the derived cell clones remained similar to that of the parental FL5.12.Bcl-X\textsubscript{L} cells following wt and mutant HA-Bax transfection, selection in hygromycin, and expression of exog-
FIG. 2. Expression and viability analysis of wild type and BH1 mutant Bax in FL5.12 clones. A, Western blot analysis of HA-tagged wt and mutant Bax and Flag-Bcl-X<sub>L</sub> proteins from FL5.12 clones were separated by 12.5% SDS-PAGE and immunoblotted with anti-HA or anti-Flag mAb. Individual cell clones were derived after transfection with appropriate gene constructs as indicated under “Experimental Procedures.” Bcl-X<sub>L</sub> represents the parental clone which was transfected with expression plasmids encoding wt or mutant Bax. Neo represents lysates from cells transfected with empty pSFFV-Neo vector. The last digit of the wt and mutant Bax:Bcl-X<sub>L</sub> cell clones indicates the clone number. Note that lysates from Bcl-X<sub>L</sub> and Neo are negative for HA-Bax, and lysates from Neo are negative for Flag-Bcl-X<sub>L</sub>. B, viability analysis of FL5.12 clones expressing Bcl-X<sub>L</sub>, Bcl-X<sub>L</sub>/wt Bax, Bcl-X<sub>L</sub>/mutant Bax, and control (Neo) treated with VP-16. Each stable transfected clone was continuously cultured with 100 μg/ml of VP-16 in the presence of IL-3. Percent viability represents the percentage of nonapoptotic cells at each time point after addition of VP-16 as measured by nuclear propidium iodide staining. All values represent the mean ± S.D. from triplicate cultures. The experiment shown is representative of at least two separate experiments. C, viability analysis of FL5.12 clones expressing Bcl-X<sub>L</sub>, Bcl-X<sub>L</sub>/wt Bax, Bcl-X<sub>L</sub>/mutant Bax,
enous Bax (Fig. 2A). Furthermore, exogenous expression of Bax and Bcl-XL did not alter the level of endogenous Bax, Bcl-XL, or Bcl-2.² We next assessed the ability of wt and mutant Bax in BH1 to antagonize the death repressor activity of Bcl-XL induced by two chemotherapy drugs, VP-16 and cisplatin (Fig. 2, B and C, respectively). Dose-response experiments determined that 100 μg/ml of VP-16 and 10 μg/ml of cisplatin were optimal for HA-Bax wt to accelerate chemotherapy-induced apoptosis when compared to the FL5.12.Flag-Bcl-XL parent cells (25) (data not shown). As seen in Fig. 2, B and C, each of the clones expressing HA-Bax Δ102–112 or HA-Bax G108 was able to counter the ability of Flag-Bcl-XL to suppress cell death as well as wt HA-Bax, when compared to the parental FL5.12.Flag-Bcl-XL cells (p < 0.01 by Student-Newman Keuls test). Therefore, Bax does not require the most conserved amino acids in BH1 to counter the death repressor activity of Bcl-XL during chemotherapy-induced cell death.

Bax Does Not Require the Most Conserved Amino Acids in BH1 to Form Heterodimers with Bcl-XL—We performed sequential immunoprecipitation and Western blot analysis of cell extracts from wt and mutant clones used in the functional assays to determine if the BH1 mutations disrupted Bax/Bcl-XL heterodimers. When lysates from these double transfected cells were immunoprecipitated with anti-Flag mAb to recognize Flag-Bcl-XL, both HA-tagged and endogenous Bax were complexed with Bcl-XL as seen on immunoblots developed with anti-Bax antibody (Fig. 3B). Both HA-Bax wt and HA-Bax G108 proteins migrated at a relative molecular mass ~24 kDa due to the addition of the HA-epitope, while the HA-Bax Δ102–112 protein migrated at the same relative molecular mass as endogenous Bax (21 kDa) due to the deletion of 11 amino acids in BH1 when resolved by 15% SDS-PAGE (Fig. 3B). As indicated by the black arrowheads in Fig. 3B, the amount of HA-Bax Δ102–112 associated with Bcl-XL was similar to the HA-Bax wt immunoprecipitated by Bcl-XL, but increased when compared to that of endogenous Bax in the adjacent lanes (white arrowhead). In reciprocal experiments, immunoprecipitation of HA-Bax using the anti-HA mAb followed by immunoblotting with anti-Bcl-X antibody revealed that Bcl-XL interacted with HA-Bax wt, HA-Bax Δ102–112, and HA-Bax G108 (Fig. 3A). Western blot analysis of total lysates from extracts used in the immunoprecipitation experiments confirmed that each clone had comparable amounts of HA-Bax wt and HA-Bax mutant protein as well as Flag-Bcl-XL protein prior to immunoprecipitation (Fig. 3, C and D). Again, the wt and mutant HA-Bax proteins migrate with a similar relative molecular mass when resolved by 12.5% SDS-PAGE using anti-HA mAb for detection of HA-Bax proteins (Fig. 3D). This demonstrates that the most conserved amino acids in BH1 are not required for Bax to form heterodimers with Bcl-XL.

Bax Does Not Require the Most Conserved Amino Acids in BH2 to Antagonize the Death Repressor Activity of Bcl-XL or to Form Heterodimers with Bcl-XL—to determine if Bax requires BH2 to counter the death repressor activity of Bcl-XL during chemotherapy-induced cell death, we generated alanine substitutions of tryptophan 151 (W151) and 158 (W158) of Bax as well as deleted amino acids 151 to 159 (Δ151–159), the most conserved residues among Bcl-2 family members in BH2 (Fig. 1C). As with BH1, the conserved amino acids in BH2 are critical for the death-repressor function of Bcl-2 and Bcl-XL (12, 16). These constructs were again introduced into the parental FL5.12.Flag-Bcl-XL cell line and clones with comparable amounts of HA-Bax wt and HA-Bax mutant protein were selected for further analysis (Fig. 4A). The BH2 mutants, HA-Bax Δ151–159, HA-Bax W151, and HA-Bax W158, were able to antagonize the ability of Bcl-XL to inhibit apoptosis induced by VP-16 and cisplatin compared to the parental FL5.12.Flag-Bcl-XL cells (p < 0.01 by Student-Newman Keuls test) (Fig. 4, B and C).

We performed sequential immunoprecipitation and immunoblotting of BH2 cell extracts used to generate the functional data to assess if the BH2 mutations disrupted Bax/Bcl-XL heterodimers. When these lysates were immunoprecipitated with anti-Flag mAb to recognize Flag-Bcl-XL, both HA-tagged and endogenous Bax were complexed with Bcl-XL as seen on immu-

² P. L. Simonian and G. Núñez, unpublished results.
FIG. 4. Expression and viability analysis of wild type and BH2 mutant Bax in FL5.12 clones. A, Western blot analysis of HA-tagged wt and mutant Bax and Flag-Bcl-X<sub>L</sub> proteins from FL5.12 clones were separated by 12.5% SDS-PAGE and immunoblotted with anti-HA or anti-Flag mAb. Individual cell clones were derived after transfection with appropriate gene constructs as indicated under “Experimental Procedures.” Bcl-X<sub>L</sub> represents the parental clone which was transfected with expression plasmids encoding wt or mutant Bax. Neo represents lysates from cells transfected with empty pSFFV-Neo vector. The last digit of the wt and mutant Bax/Bcl-X<sub>L</sub> cell clones indicates the clone number. Notice that lysates from Bcl-X<sub>L</sub> and Neo are negative for HA-Bax, and lysates from Neo are negative for Flag-Bcl-X<sub>L</sub>. B, viability analysis of FL5.12 clones expressing Bcl-X<sub>L</sub>, Bcl-X<sub>L</sub>/wt Bax, Bcl-X<sub>L</sub>/mutant Bax, and control (Neo) treated with VP-16. Each stable transfected clone was continuously cultured with 100 μg/ml VP-16 in the presence of IL-3. Percent viability represents the percentage of nonapoptotic cells at each time point after addition of VP-16 as measured by nuclear propidium iodide staining. All values represent the mean ± S.D. from triplicate cultures. The experiment shown is representative of at least two separate experiments. C, viability analysis of FL5.12 clones expressing Bcl-X<sub>L</sub>, Bcl-X<sub>L</sub>/wt Bax, Bcl-X<sub>L</sub>/mutant Bax, and control (Neo) treated with cisplatin. Each stable transfected clone was continuously cultured with 10 μg/ml cisplatin in the
protein as well as Flag-Bcl-XL protein prior to immunoprecipitation experiments confirmed that these clones to counter the death represser activity of Bcl-XL. As with BH1 and BH2 mutants, we introduced these BH3 mutants into the FL5.12.Flag-Bcl-XL, parent cell line and selected clones with similar levels of mutant Bax compared to wt Bax expression (Fig. 6A). Using the same chemotherapy-induced cell death model as before, we tested the ability of the BH3 mutant clones to counter the death inhibitory activity of Bcl-XL. We found that both Bax GD67–68 and Δ63–71 were able to accelerate VP-16 and cisplatin induced cell death compared to that observed in the parental FL5.12.Flag-Bcl-XL cells (p < 0.01 by Student-Newman Keuls test) (Fig. 6, B and C). Interestingly, the HA-Bax GD67–68.4 clone was able to accelerate chemotherapy-induced cell death faster than clone HA-Bax GD67–68.1. Densitometry comparisons revealed that HA-Bax GD67–68.4 expressed 2.5 times more mutant protein than clone HA-Bax GD67–68.1 suggesting that the amount of Bax relative to Bcl-XL is important in countering the activity of Bcl-XL (Fig. 6, A–C). In control experiments, a FL5.12.Flag-Bcl-XL clone that lacked detectable expression of HA-Bax Δ63–71 after transfection and selection in hygromycin showed the same ability as the parental FL5.12.Flag-Bcl-XL cells to inhibit chemotherapy-induced cell death indicating that the expression of wt or mutant Bax was required to antagonize Bcl-XL (data not shown). Therefore, Bax does not require BH3 to antagonize the ability of Bcl-XL during chemotherapy-induced cell death.

**Bax Requires BH3 to Form Heterodimers with Bcl-XL**—To determine the ability of Bax to associate with Bcl-XL through BH3, we performed sequential immunoprecipitation and Western blot analysis using the cell extracts from the clones assessed in the functional studies. When these lysates were immunoprecipitated with anti-Flag mAb to recognize Flag-Bcl-XL, both HA-Bax wt and HA-Bax GD67–68 as well as endogenous Bax were complexed with Bcl-XL as seen on immunoblots developed with anti-Bax antibody (Fig. 7B). As indicated by the black arrowheads in Fig. 7B, the amount of 21-kDa Bax protein immunoprecipitated by Flag-Bcl-XL was similar to the levels of endogenous Bax as seen in the adjacent lanes indicating that HA-Bax Δ63–71 did not associate with Flag-Bcl-XL. HA-Bax Δ63–71 migrates at a relative molecular mass similar to that of endogenous Bax by 15% SDS-PAGE. To verify these results, we performed reciprocal immunoprecipitation experiments using anti-HA mAb to recognize wt and mutant HA-Bax, followed by a Western blot with anti-Bcl-X antibody to detect Flag-Bcl-XL, both HA-Bax wt and HA-Bax GD67–68 clearly bound to Flag-Bcl-XL (Fig. 7A). Western blot analysis of total lysates of the same clones confirmed that each double transfected clone had similar expression of HA-Bax wt, HA-Bax Δ63–71, and HA-Bax GD67–68 protein and similar levels of Flag-Bcl-XL protein in each clone by 12.5% SDS-PAGE (Fig. 7, C and D). These results demonstrate that Bax required BH3 to associate with Bcl-XL.

**Fig. 5. Protein Interactions between Bcl-XL and wt and BH2 mutant Bax proteins in FL5.12 clones.** A, cell extracts (9 × 10⁶ cells) were immunoprecipitated with anti-HA mAb, separated by 15% SDS-PAGE, and immunoblotted with anti-HA antibody. C1 represents immunoprecipitation of lysates from Bcl-XL:Bax wt-13 cells with a nonspecific IgG2b (isotype-matched) mAb. C2 represents total lysate from FL5.12 transfected with a Flag-bcl-XL expression plasmid as a positive control. The relative molecular mass of the detected proteins is shown on the left in kDa. B, cell extracts (9 × 10⁶ cells) were immunoprecipitated with anti-Flag mAb separated on a 15% SDS-PAGE and immunoblotted with anti-Bax antibody. C1 represents immunoprecipitation of lysates from Bcl-XL:Bax wt-13 cells with a nonspecific IgG1 (isotype-matched) mAb. C3 and C4 represent total lysates from FL5.12 cells stably transfected with wt HA-tagged bax and untagged Bax expression plasmids, respectively. Black arrowheads mark HA-tagged Bax Δ151–159 protein which migrated at 21 kDa due to the deletion of 9 residues in BH2. The white arrowhead marks migration of both HA-tagged Bax Δ151–159 and endogenous Bax proteins. The relative molecular mass of the detected proteins is shown on the left in kDa. C, Western blot analysis of lysates (1 × 10⁶ cells) from the cell extracts used in the immunoprecipitation experiments depicted in A using anti-Flag mAb. C1, C2, and C3 are control lysates from FL5.12 cells stably transfected with Flag-bcl-XL and HA-bax, Flag-bcl-XL, and HA-bax expression plasmids, respectively. The molecular mass of the detected proteins is shown on the left in kDa. D, lysates (1 × 10⁶ cells) from the cell extracts used in the immunoprecipitation experiments depicted in B were separated by 12.5% SDS-PAGE and immunoblotted using anti-HA mAb. C1, C2, and C3 are control lysates as in C. The relative molecular mass of the detected proteins is shown on the left in kDa.

Noblots developed with anti-Bax antibody (Fig. 5B). HA-Bax wt, HA-Bax W151, and HA-Bax W158 proteins migrated at a relative molecular mass ~24 kDa due to the addition of the HA-epitope while the HA-Bax Δ151–159 protein migrated at the same relative molecular mass as endogenous Bax (21 kDa) due to the deletion of nine amino acids in BH2 when resolved by 15% SDS-PAGE (Fig. 5B). As indicated by the black arrowheads in Fig. 5B, the amount of HA-Bax Δ151–159 associated with Bcl-XL was similar to the HA-Bax wt immunoprecipitated by Bcl-XL, but increased when compared to that of endogenous Bax in the adjacent lanes (white arrowhead). In reciprocal experiments, immunoprecipitation of HA-Bax using the anti-HA mAb followed by Western blot analysis with anti-Bcl-X antibody revealed that Bcl-XL interacted with HA-Bax wt, HA-Bax W151, HA-Bax W158, and HA-Bax Δ151–159 (Fig. 5A). Western blot analysis of total lysates from extracts used in the immunoprecipitation experiments confirmed that these clones had comparable amounts of HA-Bax wt and HA-Bax mutant protein as well as Flag-Bcl-XL protein prior to immunoprecipitation when resolved by 12.5% SDS-PAGE using anti-HA mAb for detection of HA-Bax and anti-Flag mAb for detection of Flag-Bcl-XL (Fig. 5, C and D). Therefore, unlike Bcl-XL, Bax does not require the most conserved amino acids in BH2 to form Bax/Bcl-XL heterodimers and to counter the death represser activity of Bcl-XL during chemotherapy-induced cell death.

**Bax Does Not Require BH3 to Antagonize the Death Repressor Activity of Bcl-XL**—We next generated alanine substitutions of glycine 67 and aspartic acid 68 (GD67–68) and deleted BH3 (Δ63–71) in Bax (Fig. 1D) to determine if Bax required BH3 to counter the death repressor activity of Bcl-XL. Western blot analysis of total lysates from extracts used in the functional studies. When these lysates were immunoprecipitated with anti-Flag mAb to recognize Flag-Bcl-XL, both HA-Bax wt and HA-Bax GD67–68 as well as endogenous Bax were complexed with Bcl-XL as seen on immunoblots developed with anti-Bax antibody (Fig. 7B). As indicated by the black arrowheads in Fig. 7B, the amount of 21-kDa Bax protein immunoprecipitated by Flag-Bcl-XL was similar to the levels of endogenous Bax as seen in the adjacent lanes indicating that HA-Bax Δ63–71 did not associate with Flag-Bcl-XL, HA-Bax Δ63–71 migrates at a relative molecular mass similar to that of endogenous Bax by 15% SDS-PAGE. To verify these results, we performed reciprocal immunoprecipitation experiments using anti-HA mAb to recognize wt and mutant HA-Bax, followed by a Western blot with anti-Bcl-X antibody to detect Flag-Bcl-XL, both HA-Bax wt and HA-Bax GD67–68 clearly bound to Flag-Bcl-XL (Fig. 7A). Western blot analysis of total lysates of the same clones confirmed that each double transfected clone had similar expression of HA-Bax wt, HA-Bax Δ63–71, and HA-Bax GD67–68 protein and similar levels of Flag-Bcl-XL protein in each clone by 12.5% SDS-PAGE (Fig. 7, C and D). These results demonstrate that Bax required BH3 to associate with Bcl-XL.

presence of IL-3. Percent viability was calculated as in B. All values represent the mean ± S.D. from triplicate cultures. The experiment shown is representative of at least two separate experiments.
FIG. 6. Expression and viability analysis of wild type and BH3 mutant Bax in FL5.12 clones. A, Western blot analysis of HA-tagged wt and mutant Bax and Flag-Bcl-X<sub>L</sub> proteins from FL5.12 clones were separated by 12.5% SDS-PAGE and immunoblotted with anti-HA or anti-Flag mAb. Individual cell clones were derived after transfection with appropriate gene constructs as indicated under “Experimental Procedures.” Bcl-X<sub>L</sub> represents the parental clone which was transfected with expression plasmids encoding wt or mutant Bax. Neo represents lysates from cells transfected with empty pSFFV-Neo vector. The last digit of the wt and mutant Bax:Bcl-X<sub>L</sub> cell clones indicates the clone number. Note that lysates from Bcl-X<sub>L</sub> and Neo are negative for HA-Bax, and lysates from Neo are negative for Flag-Bcl-X<sub>L</sub>. B, viability analysis of FL5.12 clones expressing Bcl-X<sub>L</sub>, Bcl-X<sub>L</sub>/wt Bax, Bcl-X<sub>L</sub>/mutant Bax, and control (Neo) treated with VP-16. Each stable transfected clone was continuously cultured with 100 μg/ml VP-16 in the presence of IL-3. Percent viability represents the percentage of nonapoptotic cells at each time point after addition of VP-16 as measured by nuclear propidium iodide staining. All values represent the mean ± S.D. from triplicate cultures. The experiment shown is representative of at least two separate experiments. C, viability analysis of FL5.12 clones expressing Bcl-X<sub>L</sub>, Bcl-X<sub>L</sub>/wt Bax, Bcl-X<sub>L</sub>/mutant Bax,
endogenous Bax proteins. The relative molecular mass of the detected tagged Bax expression plasmids, respectively. Black arrowheads depict expected HA-tagged Bax and untagged Bax expression plasmids, respectively. Black arrowheads depict expected HA-tagged Bax Δ63–71 protein. The white arrowhead marks migration of endogenous Bax proteins. The relative molecular mass of the detected proteins is shown on the left in kDa. B, cell extracts (9 × 10⁶ cells) were immunoprecipitated with anti-Flag mAb, separated on a 15% SDS-PAGE, and immunoblotted with anti-Bax antibody. C1 represents immunoprecipitation of lysates from Bcl-XL:Bax wt-13 cells with a nonspecific IgG2b (isotype-matched) mAb. C2 and C4 represent total lysates from FL5.12 cells stably transfected with wt HA-tagged bax and untagged bax expression plasmids, respectively. Black arrowheads depict expected HA-tagged Bax Δ63–71 protein. The white arrowhead marks migration of endogenous Bax proteins. The relative molecular mass of the detected proteins is shown on the left in kDa. C, Western blot analysis of lysates (1 × 10⁶ cells) from the cell extracts used in the immunoprecipitation experiments depicted in A using anti-FLAG mAb. C1, C2, and C3 are control lysates from FL5.12 cells stably transfected with Flag-bcl-xL and HA-bax, Flag-bcl-xL and HA-hox expression plasmids, respectively. The molecular mass of the detected proteins is shown on the left in kDa. D, lysates (1 × 10⁶ cells) from the cell extracts used in the immunoprecipitation experiments depicted in B were separated on a 12.5% SDS-PAGE and immunoblotted using anti-HA mAb. C1, C2, and C3 are control lysates as in C. The relative molecular mass of the detected proteins is shown on the left in kDa.

**DISCUSSION**

In the present study, we demonstrate that Bax can antagonize the death-suppressing activity of Bcl-XL. More importantly, we provide evidence that the interaction between Bax and Bcl-XL is not required for Bax to exert its death-accelerating activity during apoptosis mediated by chemotherapeutic drugs. This indicates that Bax can antagonize Bcl-XL and promote cell death independently of its association with Bcl-XL. However, Bax was unable to completely abrogate the protection afforded by Bcl-XL upon induction of cell death as previously reported for Bcl-2 (15). A possible explanation for these findings is that the levels of Bax expressed by the cells were not high enough to completely reverse the protection afforded by Bcl-XL or Bcl-2. We have found, in additional experiments involving chemotherapeutic drugs, that the ability of wt Bax to counter the death repressor activity of Bcl-XL is enhanced with increasing amounts of Bax relative to that of Bcl-XL.² If Bax accelerated cell death by inhibiting the ability of Bcl-XL to repress cell death through direct protein interactions, we would expect that disruption of Bax/Bcl-XL heterodimerization would result in loss of acceleration by Bax. Conversely, if Bcl-XL blocked cell death by inhibiting a death-promoting activity of Bax, we would expect that disruption of the Bax/Bcl-XL heterodimer would result in complete abrogation of the ability of Bcl-XL to block cell death. The finding that Bax did not completely abrogate the death-repressor activity of Bcl-XL suggests a model whereby the death-promoting Bcl-2 homologue Bax can block the protective effects of Bcl-2 and Bcl-XL through sequestration or competition for downstream cellular factors. Bax and Bcl-XL could compete directly for a common downstream molecule or indirectly through intermediate factors. Analysis of mutations in the BH1 and BH2 conserved regions of Bcl-XL revealed that the interaction between Bcl-XL and Bax is not required for Bcl-XL to protect against apoptosis induced by IL-3 withdrawal (16). Together with our studies, the available data suggest that cell death can be regulated by Bcl-2 homologues at two distinct levels. First, death-promoting members such as Bax and death-suppressing members such as Bcl-XL can effect cell death independently perhaps through competition for downstream cellular factors. Second, apoptosis can be controlled through heterodimerization between death-accelerating and death-repressing proteins such as Bax and Bcl-XL. The Bax/Bcl-XL interaction would result in the sequestration of free Bax or free Bcl-XL and either acceleration or inhibition of apoptosis depending on level of expression of each protein. This second mechanism considers Bax/Bcl-XL heterodimers as non-functional molecules as previously proposed for Bax and Bcl-2 (15, 26). Alternatively, Bax may act upstream of Bcl-XL and promote apoptosis by enhancing a cell death signal which is inhibited by Bcl-XL or potentially, exogenous expression of Bax or Bcl-XL may induce endogenous expression of other known or yet undiscovered Bcl-2 family members. However, exogenous expression of Bax and Bcl-XL did not alter the endogenous levels of Bcl-2, Bcl-XL, or Bax when compared to the Neo control (data not shown).

The present studies indicate that Bax, like Bak requires BH3 but not BH1 or BH2 to heterodimerize with Bcl-XL. Similarly, a region of Bax encompassing 28 amino acids that contained BH3 was sufficient to interact with Bcl-2 in yeast (27). Our analysis showed that the BH3 domain of Bax is not necessary for Bax to promote chemotherapeutic-induced apoptosis that is inhibited by Bcl-XL. Therefore, in our experimental system, Bax did not require BH3 or interaction with Bcl-XL to promote apoptosis. In another study, however, Bak and Bax required BH3 to interact with Bcl-XL and also to induce cell death by transient transfection assays using Rat-1 fibroblasts (13). Several possibilities may explain the apparent discrepancy between the present results and those obtained by other investigators. First, the BH3 deletion mutant of Bax or Bak may have a differential effect on cell death depending on the cell line used for study. For example, the observation that Bak can act as both facilitator and inhibitor of cell death suggests that the cellular context of expression plays a role in determining biological activity (19). Second, our analysis was performed in stable cell lines where induction of cell death required a specific apoptotic stimulus whereas the function of Bak and Bax in previous studies was determined in transient assays where the amount of proteins expressed by the cells could not be determined. It is possible, therefore, that the type of apoptotic signal and control (Neo) treated with cisplatin. Each stable transfected clone was continuously cultured with 10 μg/ml cisplatin in the presence of IL-3. Percent viability was calculated as in B. All values represent the mean ± S.D. from triplicate cultures. The experiment shown is representative of at least two separate experiments.
or the level of gene expression is critical for the biological function observed in various systems. In agreement with our studies, other investigators have been able to develop stable cell lines after transfection of bax constructs into mammalian cells indicating that in certain cell lines, Bax overexpression does not induce cell death in the absence of a cell death signal (15).

We show in this report that Bax does not require BH1, BH2, or BH3 to exert it death-promoting activity while Bcl-2 and Bcl-XL require BH1 and BH2 for function. Bax, Bak and Bik, another putative member of the Bcl-2 family that promotes cell death (28), share the conserved BH3 region. Importantly, Bcl-2 family members such as Bcl-2 and Bcl-XL that exhibit death suppressor activity also contain conserved amino acids in BH3 indicating that the death-accelerating properties of Bax and Bak cannot be explained solely by the presence of BH3. Although Bax, Bak, Bcl-XL, and Bcl-2 all contain BH1–3 domains, they appear to require different regions and amino acids for function and heterodimerization. When the conserved glycine in BH1 of Bcl-2 (glycine 145) is changed to an alanine, the Bax/Bcl-2 association is disrupted and Bcl-2 no longer can protect from cell death (12). Mutations of amino acids 138 to 140 in BH1 of Bcl-XL also disrupted the Bax/Bcl-XL association as well as the ability of Bcl-XL to block apoptosis (16). However, in our analysis, Bax neither requires glycine 108 nor the most conserved amino acids in BH1 to promote cell death inhibited by Bcl-XL and to heterodimerize with Bcl-XL. In BH2, deletion of tryptophan 185 along with the adjoining 3 amino acids in Bcl-2 not only abrogated the ability of Bcl-2 to repress cell death induced by growth factor withdrawal, gamma irradiation, and glucocorticoids, but also disrupted heterodimerization between Bax and Bcl-2 (12). Tryptophan 188 and aspartic acid 189 when changed to glycine and alanine, reduced the ability of Bcl-XL to delay cell death, but did not completely disrupt the Bax/Bcl-XL heterodimerization (16). In our studies we neither required the conserved tryptophans 151 and 158 nor the most conserved amino acids in BH2 to promote cell death inhibited by Bcl-XL and to heterodimerize with Bcl-XL. Although both BH1 and BH2 domains are required for Bcl-XL and Bcl-2 death-repressor function, Bax does not require BH1 or BH2 to antagonize the ability of Bcl-XL to repress cell death or to heterodimerize with Bcl-XL in mammalian cells. Thus, Bax, Bcl-XL, and Bcl-2 appear to have different functional requirements for each one of the conserved domains as well as conserved amino acids within each domain. These observations suggest a model whereby different conserved regions of Bcl-2 homologues interact in a nonsymmetrical manner between certain members of the Bcl-2 family of proteins. The precise intramolecular and intermolecular structure of Bcl-2 family members are presently unresolved and need to be addressed in future studies.

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