Bax Homodimerization Is Not Required for Bax to Accelerate Chemotherapy-induced Cell Death*

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Bax, a member of the Bcl-2 family of proteins, has been shown to accelerate apoptosis induced by growth factor withdrawal, γ-irradiation, and the chemotherapeutic agent, etoposide. The mechanism by which Bax promotes apoptosis is poorly understood. Bax forms homodimers which have been suggested to act as accelerators or inducers of cell death. However, the requirement for homodimerization of Bax to promote cell death remains unclear. We performed site-directed mutagenesis of the BH1, BH2, and BH3 in Bax to determine the regions of Bax required for homodimerization and to define the role of Bax homodimers in cell death induced by chemotherapy drugs. Bax proteins expressing alanine substitutions of the highly conserved amino acids glycine 108 (G108) in BH1, tryptophan 158 (W158) in BH2, and glycine 67 and aspartic acid 68 (GD67–68) in BH3 as well as deletion of the most conserved amino acids in BH1 (Δ102–112) and BH2 (Δ151–159) and deletion of BH3 (Δ63–71) maintained their ability to accelerate chemotherapy-induced cell death. Immunoprecipitation studies revealed that Bax with deletions in BH1 and BH2 still associated with wild-type Bax while deletion of BH3 disrupted Bax homodimerization. These results demonstrate that Bax does not require the conserved regions of homology, BH1, BH2, or BH3, to accelerate chemotherapy-induced cell death. Furthermore, our results established BH3 as a region required for Bax homodimerization in mammalian cells and demonstrate that monomeric forms of Bax are active in accelerating cell death induced by chemotherapy agents.

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 and repressors (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 domains 1, 2, and 3 (BH1, BH2, and BH3) (12, 13). Bax, another Bcl-2 family member, counters the death repressor activity of both Bcl-2 and Bcl-XL and accelerates cell death induced by growth factor withdrawal (14) and the chemotherapy drug, etoposide (15). The precise mechanism whereby Bax antagonizes the ability of Bcl-2 to repress cell death and accelerates cell death remains unclear. Previous studies suggested that Bax homodimers are the functional form involved in acceleration of cell death (14, 16). It has been proposed that Bax homodimers promoted cell death while Bcl-2 homodimers function as repressors of cell death (14, 16). These observations prompted a model in which susceptibility to cell death depends upon the relative amount of Bcl-2 and Bax homo- and heterodimers present in the cell (16). However, the mechanism by which Bax accelerates cell death and the role of Bax homodimers in promoting cell death remains unclear.

In this report, we performed site-directed mutational analysis of the conserved BH1, BH2, and BH3 of Bax to determine their role in Bax homodimerization and to define the requirement for Bax homodimers in cell death. Our studies demonstrate that Bax does not require BH1, BH2, or BH3 to accelerate chemotherapy-induced cell death. Furthermore, our results define the conserved BH3 domain of Bax as a region required for homodimerization and demonstrate that Bax homodimers are not necessary for acceleration of cell death induced by chemotherapy agents.

**EXPERIMENTAL PROCEDURES**

*Generation of bax Constructs—An influenza virus hemagglutinin (HA)1 epitope was introduced at the amino terminus of Bax by PCR amplification using a murine bax cDNA template previously described (14). HA-tagged bax insert was subcloned into the EcoRI site of pSFFV-Neo expression vector (14). Proper orientation was determined by restriction mapping, and the authenticity of the inserts was confirmed by dideoxy sequencing. The specific alanine mutations and deletions in the BH1, BH2, and BH3 of Bax were constructed by a two-step PCR mutagenesis method as described (17, 30). The resulting PCR product was evaluated for correct size on a 1% agarose gel, purified, and subcloned into the EcoRI site of the pSFFV-Neo expression vector. Orientation of the inserts was determined by restriction mapping. The fidelity of all

1 The abbreviations used are: HA, hemagglutinin; IL-3, interleukin 3; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; wt, wild-type; mAb, monoclonal antibody.
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Fig. 1. Schematic representation and expression of wild-type and mutant Bax in FL5.12 clones. A, shaded rectangles of Bel-2 homology domains are designated BH1, BH2, and BH3. Numbers indicate position of residues which define these homology regions in wild-type Bax (top). The deletion or alanine substitution for each mutant generated is indicated. B, Western blot analysis of HA-tagged wild and mutant Bax proteins from FL5.12 clones using anti-HA mAb. Individual cell clones were derived after transfection with appropriate gene constructs as indicated under “Experimental Procedures.” Neo represents lysates from cells transfected with pSFFV-Neo vector alone. The last digit of the wild type and mutant Bax cell clones indicates the clone number. Note that the lysate from Neo was negative for HA.

mutant HA-tagged bax constructs was confirmed by dideoxy sequencing. The schematic representation of each mutation generated in Bax is provided in Fig. 1A. A lacZ expression plasmid was constructed by subcloning the lacZ open reading frame into the EcoRI site of the pSFFV-Neo expression vector as described above.

Cell Culture and Transfection—The pro-B cell line, FL5.12, was cultured in interleukin-3 (IL-3) conditioned medium as described (18). FL5.12 were transfected by electroporation (200 V, 960 microfarads) with 10 μg of the pSFFV-Neo plasmid containing each HA-bax mutant construct in the sense orientation, lacZ, or control pSFFV-Neo plasmid. Individual cell clones were selected for growth in the presence of G418 (1.0 mg/ml) by limiting dilution. Expression of HA-bax in single cell clones was analyzed by flow cytometry using anti-HA (clone 12CA5) (Boehringer Mannheim) as described (19), and protein expression was confirmed by Western blot analysis as described below. Human embryonic kidney cells (293T cells) were maintained in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc.) and transfected (10 μg for each construct) using calcium phosphate (20).

Western Blot and Immunoprecipitation Analysis—The expression of HA-Bax was determined by Western blot analysis as described previously (19). Briefly, 1 × 10^7 cells were lysed in Nonidet P-40 isotonic lysis buffer (142.5 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.2), 1 mM EGTA, 0.2% Nonidet P-40) with freshly added protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.7 μg/ml pepstatin, and 1 μg/ml leupeptin) as described (14) and electrophoresed through 20% SDS-polyacrylamide gels, transferred to nitrocellulose, blocked with 5% nonfat dry milk (Nestle Food Co., Glendale, CA), and incubated with anti-HA mAb (1 μg/ml), rabbit polyclonal anti-Bax (1:1000 dilution), or anti-β-tubulin mAb (clone TUB 2.1) (Sigma) to assess equal protein loading. Rabbit antiserum to Bax was raised against a synthetic peptide corresponding to residues 4–19 of human Bax, coupled with bis-diazobenzidine to keyhole limpet hemocyanin. After incubation with secondary antibody, the reaction was developed by enhanced chemiluminescence using the ECL kit (Amersham) and exposed to film (Eastman Kodak) for an average of 2 min. For FL5.12 immunoprecipitations, 1 × 10^7 cells were lysed in Nonidet P-40 isotonic lysis buffer (14) and rotated with anti-HA mAb (10 μg/ml) for 90 min at 4 °C. 10% (v/v) of goat anti-mouse IgG (H + L) Sepharose B (Zymed Laboratories Inc., San Francisco, CA) was added for an additional hour of incubation by rotation. Immune complexes were centrifuged and washed with cold Nonidet P-40 isotonic lysis buffer at least three times, separated by 20% SDS-PAGE, and immunoblotted using rabbit anti-Bax as described above. 293T cells were transiently transfected with calcium phosphate as described (20) with 10 μg of each construct in 10-mm plates, lysed, and immunoprecipitated as described above 36 h after transfection. Lysate from one-tenth of the 293T total cellular extract was used for Western blot analysis as described above.

Chemotherapy-induced Cell Death Assays—Cells were seeded at 1 × 10^6 cells per 10-cm plate in 1 ml of high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. The experiment shown is representative of at least two individual experiments.
10^5 cells/ml in triplicate wells in media containing IL-3 and either taxol (0.01 μg/ml) (Mead Johnson, Princeton, NJ), vincristine (0.1 μg/ml) (Eli Lilly, Indianapolis, IN), cisplatin (10 μg/ml) (Bristol Laboratories, Princeton, NJ), VP-16 (20 μg/ml) (Gensia Laboratories, LTD., Irvine, CA), or fluorouracil (10 μg/ml) (Hoffman-La Roche, Nutley, NJ). The percentage of apoptotic cells was determined at different time points in triplicate cultures by nuclear propidium iodide staining followed by flow cytometric analysis as described previously (19). In this assay, the nuclei of apoptotic cells exhibit a sub-G0 profile characteristic of DNA fragmentation (21). Analysis was performed using Lysys II software on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Resultswere based on the analysis of at least 5 × 10^4 events from each triplicate culture. Values were expressed as the mean ± S.D. from triplicate cultures. The experiment shown is representative of at least two individual experiments.

RESULTS AND DISCUSSION

We generated mutations and deletions of the most conserved amino acids in BH1, BH2, and BH3 domains in Bax to determine their requirement for Bax activity and Bax homodimerization (Fig. 1A). In BH1, glycine 108 (G108) was changed to alanine or residues 102 to 112 (Δ102–112) were deleted. These amino acids in BH1 were required for Bcl-2 or Bcl-XL to form heterodimers with Bax and for their anti-apoptotic activity against IL-3 withdrawal (12, 22). In BH2, we substituted tryptophan 158 with alanine and deleted residues 151 to 159 (Δ151–159). As with BH1, these conserved residues in BH2 were critical for the death-repressor activity of Bcl-2 and Bcl-XL (12, 22). We next generated alanine substitutions of glycine 67 and aspartic acid 68 (GD67–68) and deleted all of the residues in BH3 (Δ63–71) (13). Constructs containing HA-bax wt, HA-bax G108, HA-bax Δ102–112, HA-bax W158, HA-bax Δ151–159, HA-bax GD 67–68, and HA-bax Δ63–71 were transfected into FL5.12 lymphoid cells. After selection in G418, two sets of clones for each construct were identified based on a similar level of mutant Bax expression compared with that of a HA-bax wt clone (Fig. 1B and data not shown).

We next assessed the ability of wt and mutant Bax proteins to accelerate cell death induced by the chemotherapy drugs taxol and vincristine. Dose-response experiments determined that 0.01 μg/ml taxol and 0.1 μg/ml vincristine were optimal for HA-Bax wt clone 13 to accelerate chemotherapy-induced cell death compared with cells transfected with the pSFFV-Neo vector alone (data not shown). As seen in Fig. 2, A and B, all of the Bax mutant proteins were able to accelerate taxol- and vincristine-induced cell death compared with the pSFFV-Neo vector alone and with different chemotherapy drugs.
control \( (p < 0.01 \text{ by Student-Newman Keuls test}) \). Analysis of a second set of independently derived clones showed results similar to those presented in Fig. 2 (data not shown). To exclude the possibility that the acceleration promoted by Bax expression was due to expression of a nonspecific protein in FL5.12 cells or selection of a cell death-resistant pSFFV-Neo control clone, we transfected control lacZ pSFFV-Neo or pSFFV-Neo alone into FL5.12 cells and isolated multiple stably transfected clones. Analysis of multiple FL5.12 cell clones expressing \( \beta \)-galactosidase or the neomycin resistance gene did not show a statistically different rate of taxol- or vincristine-induced cell death when compared to the pSFFV-Neo control clone shown in Fig. 2, while HA-bax wt clone 13 accelerated cell death in the same experiments (data not shown). These results indicate that the most conserved amino acids in BH1, BH2, or BH3 are not necessary for Bax to promote chemotherapy-induced apoptosis in FL5.12 cells. However, other studies suggested that the BH3 conserved region of Bax was required for induction of cell death in transient transfection assays using fibroblast cell lines (13, 23). Therefore, to further verify our results, we isolated three additional HA-bax \( \Delta 63–71 \) FL5.12 clones 8, 11, and 17 with levels of protein expression similar to HA-Bax \( \Delta 63–71 \) clone 10 (Fig. 3A). These additional HA-Bax \( \Delta 63–71 \) mutant clones were able to accelerate cell death induced by taxol and vincristine to the same extent as HA-Bax \( \Delta 63–71 \) clone 10 (data not shown). To strengthen these results, we assessed the ability of these four HA-Bax \( \Delta 63–71 \) clones to accelerate cell death induced by three additional chemotherapeutic drugs. As seen in Fig. 3B, all four HA-Bax \( \Delta 63–71 \) FL5.12 clones exhibited a similarly increased rate of cell death compared with the pSFFV-Neo control clone when treated with cisplatin, etoposide, and fluorouracil \( (p < 0.01 \text{ by Student-Newman Keuls test}) \).

Several studies have suggested that Bax homodimers are the functional form of Bax that promote cell death (14, 16). Consequently, the ability of Bax mutants to form homodimers was determined by sequential immunoprecipitation and immunoblotting using cell extracts from the clones assessed in the functional studies. As seen in Fig. 4A, immunoprecipitation of HA-tagged mutant Bax using anti-HA mAb showed that mutations in the BH1 and BH2 conserved regions of Bax retained the ability of Bax to associate with endogenous Bax protein. However, mutant HA-Bax \( \Delta 151–159 \) had a decreased ability to interact with endogenous Bax protein when compared to HA-Bax wt (Fig. 4A, top panel). This was not due to different amounts of Bax protein expressed in HA-Bax \( \Delta 151–159 \) transfectedants since the levels of endogenous Bax were not affected by transfection with HA-bax wt, mutant HA-bax, or pSFFV-Neo constructs and selection in G418 (Fig. 4A, bottom panel). Deletion of BH3, however, disrupted the ability of Bax to form homodimers with endogenous Bax (Fig. 4A, top panel). To determine if mutant Bax \( \Delta 63–71 \) protein could form homodimers, an untagged bax \( \Delta 63–71 \) construct was generated and transiently co-transfected with HA-tagged bax \( \Delta 63–71 \) into 293T cells. As seen in Fig. 4B, immunoprecipitation of wt and mutant \( \Delta 63–71 \) Bax using the HA-specific mAb followed by immunoblotting with anti-Bax antibody revealed that HA-Bax wt associated with endogenous Bax, but HA-Bax \( \Delta 63–71 \) did not interact with endogenous Bax or untagged Bax \( \Delta 63–71 \). Western blot analysis of total lysates from cell extracts used in the immunoprecipitation experiments showed expression of HA-Bax wt, HA-Bax \( \Delta 63–71 \), and untagged Bax \( \Delta 63–71 \) as well as endogenous Bax (Fig. 4C). This demonstrates that the BH3 domain of Bax is required for Bax homodimerization and that mutant Bax \( \Delta 63–71 \) cannot form homodimers. Together with the results presented in Figs. 2 and 3, these findings demonstrate that Bax homodimerization is not necessary for Bax to accelerate chemotherapy-induced cell death.

In the present studies, the most conserved amino acids in BH1 or BH2 of Bax were not required to promote chemotherapy-induced cell death. Although both BH1 and BH2 domains are necessary for Bel-X\(_L\) and Bel-2 death repressor function, Bax did not require BH1 or BH2 to accelerate taxol- and vincristine-induced cell death. Moreover, Bik and Bel-X\(_L\), which do not contain BH1 or BH2 and exhibit weak homology in BH3 also promoted cell death suggesting that BH1 and BH2 are not required for these Bel-2-related proteins to accelerate cell death (24, 25). In recent reports, however, BH3 was necessary for Bax to induce cell death by transient transfection assays using Rat-1 and human GM701 fibroblasts (13, 23). Several possibilities may explain the apparent discrepancy between the present results and those obtained by other investigators.
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First, the BH3 deletion mutant of Bax may have a differential effect on cell death depending on the cell line used for study. For example, the observation that Bax can act as both facilitator and inhibitor of cell death suggests that the cellular context of expression plays a role in determining biological activity (26, 27). Second, our analysis was performed in stable cell lines where induction of cell death required a specific apoptotic stimulus whereas the function of Bax in previous studies (13, 23) was determined in transient assays where the amount of protein 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 or the level of gene expression is critical for the biological function observed in various systems.

Members of the Bcl-2 family such as Bcl-2 or Bcl-XL inhibit apoptosis, whereas others that include Bax and Bak function as death-accelerating proteins. Susceptibility of a cell to apoptotic signals appears to be regulated in part by the relative levels and competing dimerizations of different Bcl-2 family members (14, 16, 28). Two nonexclusive models have been proposed to explain the role of Bax in cell death. Bax could exhibit intrinsic death-accelerating activity, and death-suppressing Bcl-2 family members may serve as dominant inhibitors. Alternatively, Bax could promote cell death by inhibiting the protective activity of Bcl-2 and functional homologues. It has been hypothesized that homodimers of Bax function as accelerators or inducers of cell death (13, 14, 16). Here we demonstrate that homodimerization of Bax is not required for Bax to accelerate cell death mediated by several chemotherapeutic agents. Our finding that BH3 of Bax is required for homodimerization is in accord with recent observations in yeast (29). Previous studies of Bax mutants have demonstrated that Bax can antagonize Bcl-XL independently of its binding with Bcl-XL (30). These observations suggested that Bax inhibits the protective activity of Bcl-XL and promotes cell death through sequestration or competition for downstream targets or the generation of cell death signals (30). Hence, these findings support a model in which apoptosis is regulated by Bcl-2 homologues at two distinct levels. First, members such as Bax promote cell death and antagonize Bcl-2 or Bcl-XL independently of their physical interactions. A second level of regulation is through heterodimerization between death-accelerating proteins such as Bax and death inhibitors such as Bcl-2. The Bcl-2-Bax heterodimer would result in reduced levels of free Bax or Bcl-2 and influence susceptibility to apoptosis depending on the relative levels of each protein. Because monomeric forms of Bax are active in promoting cell death, our results suggest that the intrinsic death-promoting activity of Bax is inactivated when dimerized with Bcl-2 or Bcl-XL.

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