Differential Regulation of Bax and Bak by Anti-apoptotic Bcl-2 Family Proteins Bcl-B and Mcl-1*

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The pro-apoptotic members of the Bcl-2 family include initiator proteins that contain only BH3 domains and downstream effector multi-BH domain-containing proteins, including Bax and Bak. In this report, we compared the ability of the six human anti-apoptotic Bcl-2 family members to suppress apoptosis induced by overexpression of Bax or Bak, correlating findings with protein interactions measured by three different methods: co-immunoprecipitation, glutathione *S*-transferase pulldown, and fluorescence polarization assays employing synthetic BH3 peptides from Bax and Bak. Bcl-B and Mcl-1 showed strong preferences for binding to and suppression of Bax and Bak, respectively. In contrast, the other anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-X_L, Bcl-W, and Bfl-1) suppressed apoptosis induced by overexpression of either Bax or Bak, and they displayed an ability to bind both Bax and Bak by at least one of the three protein interaction methods. Interestingly, however, full-length Bax and Bak proteins and synthetic Bax and Bak BH3 peptides exhibited discernible differences in their interactions with some anti-apoptotic members of the Bcl-2 family, cautioning against reliance on a single method for detecting protein interactions of functional significance. Altogether, the findings reveal striking distinctions in the behaviors of Bcl-B and Mcl-1 relative to the other anti-apoptotic Bcl-2 family members, where Bcl-B and Mcl-1 display reciprocal abilities to bind and neutralize Bax and Bak.

Mitochondria are primary centers for apoptosis control within mammalian cells (1, 2). Bcl-2 family proteins are key regulators of mitochondrial-related apoptosis pathways and function to either promote or suppress mitochondrial outer membrane permeabilization, thereby providing a checkpoint for the release into the cytosol of mitochondrial proteins responsible for downstream apoptotic mechanisms (3–5).

The anti-apoptotic Bcl-2 proteins all contain four conserved Bcl-2 homology (BH)[2] domains, BH1 to BH4. To date, six human anti-apoptotic Bcl-2 family proteins have been

reported, including Bcl-X_L, Bcl-2, Bcl-W, Bcl-B, Bfl-1, and Mcl-1 (6). The pro-apoptotic Bcl-2 family members are divided into two groups, the BH3-only and multidomain groups. The BH3-only proteins usually act as initial apoptosis signal sensors. More than 12 BH3-only proteins have been reported (*e.g.* Bid, Bad, Bim, Noxa, and Puma), and they convey different stress signals to the core cell death machinery (7–10). The multidomain Bcl-2 family proteins (Bax, Bak, and perhaps Bok) contain BH1, BH2, and BH3 domains and function to promote mitochondrial leakage if not suppressed by the binding of anti-apoptotic Bcl-2 family members (11). BH3-only proteins may activate Bax and Bak directly or indirectly by suppressing the function of anti-apoptotic Bcl-2 family proteins (8–10). Furthermore, anti-apoptotic Bcl-2 family proteins may nullify the function of the BH3-only proteins by binding and inhibiting the downstream interactions of BH3-only proteins with Bax and Bak (4).

Experiments with Bax/Bak double knock-out mice showed that Bax and Bak are downstream checkpoint proteins that are capable of regulating the permeability of the mitochondrial outer membrane (11). Interactions between anti-apoptotic Bcl-2 family proteins and Bax or Bak, therefore, constitute a final step in determining mitochondria-induced cell death. Thus, it is of interest to elucidate the functional and physical interactions among anti-apoptotic members of the Bcl-2 family with Bax and Bak.

For this reason, we tested the ability of all six human anti-apoptotic Bcl-2 family proteins to suppress apoptosis induced by Bax or Bak and correlated these results with protein binding, using immunoprecipitation (IP), GST pulldown, and fluorescence polarization assays (FPAs). Of the six anti-apoptotic Bcl-2 family members, Bcl-B and Mcl-1 showed clear preferences for binding to and suppressing apoptosis induced by Bax and Bak, respectively, whereas the other family members (Bcl-2, Bcl-X_L, Mcl-1, and Bcl-W) were far less selective. Thus, Bcl-B and Mcl-1 stand apart from the other anti-apoptotic members of the Bcl-2 family, suggesting that these proteins have specific roles in the suppression of cell death pathways that predominantly involve Bax or Bak, respectively.

MATERIALS AND METHODS

Plasmid Constructions—Plasmids encoding Myc-tagged, anti-apoptotic human Bcl-2 family proteins were cloned into the EcoRI and XhoI sites of the pcDNA3-Myc vector as previ-

4,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; HPLC, high pressure liquid chromatography; AFC, 7-amino-4-trifluoromethyl coumarin.

ously reported (12). GFP-Bax and GFP-Bak plasmid clones have previously been described (13).

Co-immunoprecipitation (co-IP) Assays—HEK293T cells were cultured in 6-well plates and were transiently transfected with 1 μg of GFP control plasmid and 1 μg of plasmids encoding GFP-Bax or -Bak, together with 1 μg of pcDNA3-Bcl-X$_L$, Bcl-2, Bcl-W, Bcl-B, Bfl-1, or Mcl-1. The cells were cultured for 20 h in 50 μM benzoyl-Val-Ala-Asp-fluoromethylketone (Bachem) to avoid apoptosis and then suspended in lysis buffer (10 mM HEPES, pH 7.4, 142.4 mM KCl, 5 mM MgCl$_2$, 0.5 mM EGTA, 2% CHAPS containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Roche Applied Science)). The cell lysates were subjected to IP using anti-Myc-coated beads incubated overnight at 4 °C. Immune complexes were analyzed by SDS-PAGE/immunoblotting using mouse monoclonal anti-GFP and -Myc antibodies (Santa Cruz, Inc., Santa Cruz, CA).

Protein Purification—The Bax and Bak proteins were produced in bacteria and purified similar to a previous report (14). Bak without transmembrane domain (1–187) was subcloned into a modified pET21b plasmid and purified as reported previously (15). GST fusion proteins containing Bcl-X$_L$, Bcl-2, Bcl-W, Bcl-B, Bfl-1, and Mcl-1 lacking the C-terminal transmembrane domains (approximately the last 20 amino acids, ΔTM) were expressed from pGEX 4T-1 plasmid in XL-1 Blue cells (Stratagene, Inc., La Jolla, CA) similar to previous reports (16, 17). Briefly, the cells were grown in 2 liters of LB media with 50 μg/ml ampicillin at 37 °C to an A_{600} nm of 1.0 followed by the addition of isopropyl β-D-thiogalactopyranoside (0.5 mM) and incubated at 25 °C for 6 h. The cells were then recovered in 20 mM phosphate buffer (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA followed by sonication. The cellular debris were removed by centrifugation at 27,500 × *g* for 20 min, and the resulting supernatants were incubated with 10 ml of glutathionine-Sepharose (Amersham Biosciences) at 4 °C for 2 h. The resin was washed three times with 20 mM phosphate buffer (pH 7.4), 150 mM NaCl, and 1 mM dithiothreitol, followed by elution of GST fusion proteins in 10 mM of reduced glutathione dissolved in 50 mM Tris-HCl (pH 8.0).

GST Pulldown Assays—GST or GST-Bcl-X$_L$, -Bcl-2, -Bcl-W, -Bcl-B, -Bfl-1, or -Mcl-1 (1 μg each) were preincubated separately with 10 μl of glutathione-Sepharose 4B resin for 2 h at 4 °C in lysis buffer (10 mM HEPES, pH 7.4, 142.4 mM KCl, 5 mM MgCl$_2$, 0.5 mM EGTA, 1 mM dithiothreitol) containing 2% CHAPS. The resins were washed once in lysis buffer, followed by the addition of 1 μg of purified Bax or Bak protein. The reactions were performed in 500 μl of lysis buffer with overnight incubation at 4 °C. The resins were washed with lysis buffer three times, boiled, and analyzed by SDS-PAGE/immunoblotting using GST (mouse monoclonal antibody made in our laboratory), Bax (N20; Santa Cruz), or Bak (06-536; Upstate) antibodies.

Cell Culture, Transfection, and Apoptosis Assays—HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and antibiotics. For transient transfection apoptosis assays, the cells at ~80% confluence (5 × 10^5) were transfected using Lipofectamine 2000 (Invitrogen) in 6-well plates with 0.5 μg of plasmids encoding

GFP or 3 μg of pcDNA3 Bcl-2, or GFP-Bax, GFP-Bak, alone, or in combination with 1, 2, or 3 μg of either pcDNA3-Bcl-X$_L$, Bcl-2, Bcl-W, Bcl-B, Bfl-1, or Mcl-1. Apoptosis was assessed by 4,6-diamidino-2-phenylindole (DAPI) staining of cells. Briefly, at 20 h after transfection, both floating and adherent cells were collected, fixed with phosphate-buffered saline containing 3.7% formaldehyde, and stained with DAPI in phosphate-buffered saline. The percentage of apoptotic cells was determined by UV microscopy, counting GFP-positive cells that displayed nuclear fragmentation and/or chromatin condensation. All of the assays were performed in triplicate. For caspase assays, cell lysates were prepared 20 h post-transfection and normalized for protein content, and 10-μg aliquots of cell lysates were incubated with 100 μM DEVD-AFC, measuring enzyme activity by the generation of AFC fluorescence. The data are reported as relative fluorescence units of product produced per minute/10 μg of total protein.

Peptide Synthesis—Fluorescein isothiocyanate (FITC)-conjugated or nonlabeled Bax and Bak BH3 peptides and FITC-Bim BH3 peptide were synthesized on an Advanced Chem Tech 350 multiple peptide synthesizer (Aapptec, Louisville, KY) using 1,3-diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) as coupling reagent for Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) solid phase peptide synthesis. The amino acid sequences of the BH3 peptides were: Bax, QDASTKKLSE-CLKRIGDELDS; Bak, PSSTMGQVGRQLAIIGDDINRRYDS; and Bim, DMRPEIWIAQELRRIGDEFNAYYAR. The crude peptides were purified with a Gilson HPLC instrument, with detection at 210 nm. Analytical reverse phase HPLC was routinely performed on a Vydac 218TP54 or 238TP54 column (C18, 4.6 × 250 mm, 5 μm) at a flow rate of 1.5 ml/min using a linear gradient of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) over 20 min (0–100% B). Preparative reverse phase HPLC was performed on a Cosmosil column (5C18-AR, 20 × 250 mm) at a flow rate of 8 ml/min using a gradient of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 90% acetonitrile. The purified peptide was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry using an Applied Biosystems Voyager System 6264 (Applied Biosystems, Foster City, CA).

Fluorescence Polarization Assays—FPAs were performed according to published procedures (13, 14). Briefly, various concentrations of GST-Bcl-2 family proteins were incubated with 10–15 nM of FITC-conjugated Bax BH3 peptide (FITC-Ahx-QDASTKKLSECLKRIGDELDS), FITC-Bak BH3 peptide (FITC-Ahx-PSSTMGQVGRQLAIIGDDINRRYDS), or FITC-Bim BH3 peptide (FITC-Ahx-DMRPEIWIAQELRRIG-DEFNAYYAR). Fluorescence polarization was measured using an Analyst™ AD assay detection system (LJL Biosystem, Sunnyvale, CA) in phosphate-buffered saline (pH 7.4) after incubating for 10 min in a 96-well black plate (Greiner Bio-One, Monroe, NC). EC$_{50}$ determinations were performed using GraphPad Prism software (GraphPad, Inc., San Diego, CA).

Competition Assays—Using the same procedure described above, 100 nM of GST-Bcl-2 family proteins were incubated with various concentrations of unlabeled Bax and Bak BH3 peptides, followed by 15 nM of FITC-Bax, 10 nM of FITC-Bak, or 15
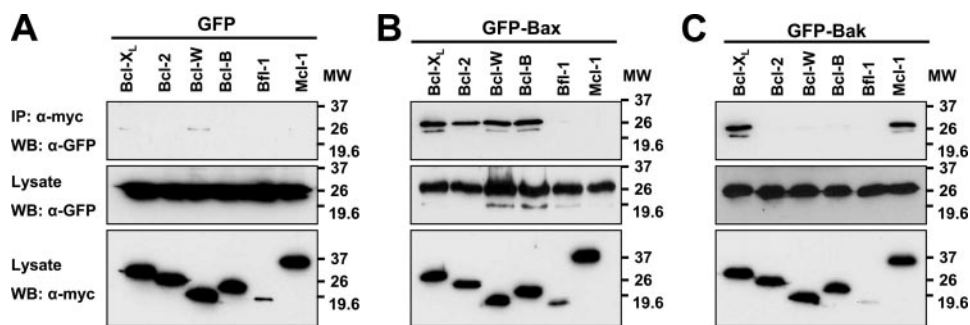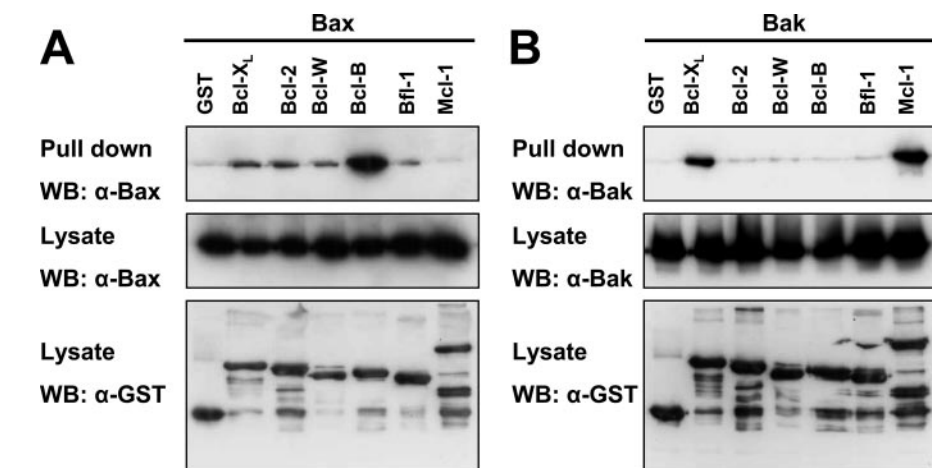
FIGURE 1. **Immunoprecipitation of anti-apoptotic Bcl-2 family proteins with Bax and Bak.** HEK293T cells were transiently transfected in 6-well dishes with 1 μg of GFP control (*A*), and 1 μg of plasmids encoding GFP-Bax (*B*) or GFP-Bak (*C*) together with 1 μg of Myc-tagged pcDNA3-Bcl-X$_L$, -Bcl-2, -Bcl-W, -Bcl-B, -Bfl-1, or -Mcl-1. Lysates were normalized for total protein concentration and then subjected to immunoprecipitation with anti-Myc antibody. Immune complexes were analyzed by SDS-PAGE/immunoblotting using mouse monoclonal GFP antibody (*top panels*). To assess expression of proteins, equivalent volumes of lysates were also loaded directly in gels and analyzed by SDS-PAGE/immunoblotting using antibodies to GFP (*middle panels*) or Myc (*bottom panels*). *IP*, immunoprecipitation; *WB*, Western blot; *MW*, molecular mass.

FIGURE 2. **GST-Bcl-2 family proteins associate with Bax and Bak *in vitro*.** Recombinant purified Bax (*A*) or Bak (*B*) proteins were incubated at 4 °C overnight with resin containing GST or GST-Bcl-X$_L$, -Bcl-2, -Bcl-W, -Bcl-B, -Bfl-1, or -Mcl-1 (1 μg each), and GST pulldown assays were performed followed by SDS-PAGE/immunoblotting using anti-Bax or -Bak antibody (*top panels*). Input proteins were loaded directly as controls (*middle* and *bottom panels*). *WB*, Western blot.

nM of FITC-Bim. Fluorescence polarization was measured after 10 min as described above. IC$_{50}$ determinations were performed using GraphPad Prism software (GraphPad, Inc.).

RESULTS

Selective Association of Anti-apoptotic Bcl-2 Family Proteins with Bax and Bak—We previously showed that human anti-apoptotic Bcl-B protein binds and regulates Bax- but not Bak-induced cell death (13). In this report, we further extend the studies to all six human anti-apoptotic Bcl-2 family proteins, including Bcl-X$_L$, Bcl-2, Bcl-W, Bcl-B, Bfl-1, and Mcl-1. To explore the binding of anti-apoptotic Bcl-2 family proteins to Bax and Bak, co-IP assays were performed following transient transfection of HEK293T cells. For these experiments, anti-apoptotic Bcl-2 family proteins were expressed with N-terminal Myc epitope tags, whereas Bax and Bak were expressed with N-terminal GFP tags. CHAPS-containing lysis solution was used, because of reports that this nonionic detergent better preserves natural conformations of Bcl-2 family proteins such as Bax (18). co-IP experiments showed that Bax binds to Bcl-X$_L$, Bcl-2, Bcl-W, and Bcl-B, but not Mcl-1. In contrast, binding of

Bak was detected by co-IP to only Bcl-X$_L$ and Mcl-1 (Fig. 1). The results regarding the ability of Bfl-1 to bind Bax or Bak were unclear because of its low expression level in these experiments. The detected interactions between the anti-apoptotic Bcl-2 family proteins and GFP-Bax or -Bak proteins were specific, in as much as no nonspecific association of GFP was observed with the anti-apoptotic Bcl-2 family proteins (Fig. 1). Immunoblot analysis of GFP, GFP-Bax, and GFP-Bak served as a loading control.

Because use of cellular lysates for co-IP experiments cannot exclude the possibility of involvement of indirect partners in the observed protein associations, we further investigated the interaction of Bcl-2 family proteins with Bax and Bak using recombinant purified proteins *in vitro*. All six anti-apoptotic Bcl-2 family proteins were expressed as N-terminal GST fusion proteins without their C-terminal transmembrane domains (~20 amino acids). GST pulldown results were similar to IP findings in that Bcl-X$_L$, Bcl-2, Bcl-W, and Bcl-B associated with Bax. We also confirmed that recombinant Bfl-1 can bind to Bax, which was not evident from the IP experiments because of poor expression (Fig. 2*A*). The results from GST pulldown assays for Bak correlated with IP results, with only Bcl-X$_L$ and Mcl-1 demonstrating associations with Bak (Fig. 2*B*). GST alone demonstrated only background binding to Bax or Bak, indicating the specificity of the binding results.

Differential Protection of Anti-apoptotic Bcl-2 Family Proteins against Cell Death Induced by Bax or Bak—To correlate protein interaction data with anti-apoptotic function, we tested the ability of the six anti-apoptotic Bcl-2 family proteins to suppress apoptosis induced by Bax and Bak overexpression. When plasmids encoding Bax and Bak were transiently transfected into HEK293T cells, roughly 90% of the transfected cells (marked by GFP) underwent apoptosis within 20 h, as determined by DAPI staining. Co-expression of Bcl-X$_L$, Bcl-W, Bcl-B, and Bfl-1, but not Mcl-1, markedly suppressed apoptosis induced by Bax in HEK293T cells, as shown by DAPI staining and caspase activity assays (Fig. 3, *A* and *C*). For Bak, co-expression of Bcl-X$_L$, Bcl-W, Bfl-1, and Mcl-1 demonstrated protection from Bak-mediated apoptosis, whereas Bcl-B did not (Fig. 3, *B* and *D*). The relatively high quantity of Bcl-2 plasmid used in these experiments (ranging from 1 to 3 μg) resulted in significant background cell death. When the quantity of Bcl-2
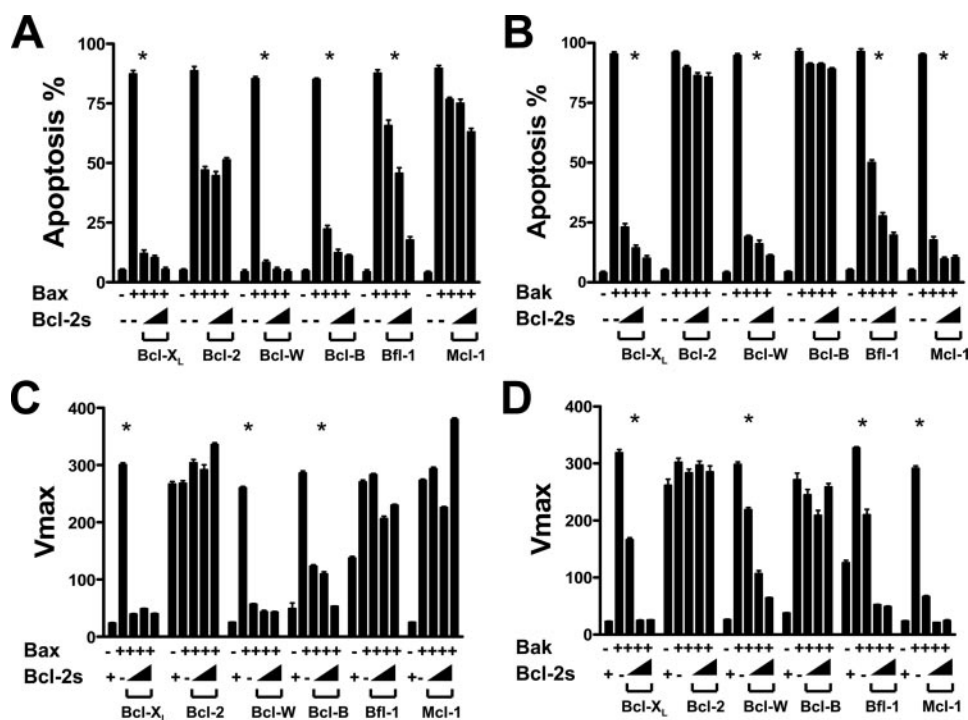
FIGURE 3. **Protection by Bcl-2 family proteins against Bax and Bak-induced cell death.** HEK293T cells were transfected at ~80% confluence in 6-well dishes with 0.5 μg of plasmids encoding GFP (*first column* of each panel) or 3 μg of pc DNA3 Bcl-2 (*first column* of each panel for *A* and *B*) or 0.5 μg of plasmid of GFP-Bax or GFP-Bak alone (*second column* of each panel) or in combination with 1, 2, or 3 μg of pcDNA3-Bcl-X$_L$, -Bcl-2, -Bcl-W, -Bcl-B, -Bfl-1, or -Mcl-1, separately (*third*, *fourth*, and *fifth* of each column of each panel). For apoptosis assays (*A* and *B*), floating and adherent cells were collected, fixed, and stained with 0.1 μg/ml DAPI. The percentages of apoptotic cells were determined by counting the GFP-positive cells displaying nuclear fragmentation and/or chromatin condensation (mean ± S.D.; n = 3). For caspase assays (*C* and *D*), the lysates were normalized for protein content (10 μg) and incubated with 100 μM Ac-DEVD-AFC. Enzyme activity was determined by the generation of fluorescent AFC product, V_{max} was calculated, and the data were expressed as relative fluorescence units/min/1 μg (mean ± S.D.; n = 3). Statistical significance was determined by analysis of variance ($p \leq 0.01$ indicated by *asterisk*).
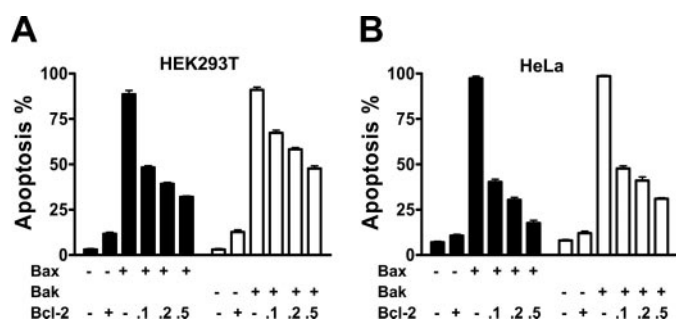
FIGURE 4. **Protection by Bcl-2 against Bax- and Bak-induced cell death.** HEK293T (*A*) or HeLa (*B*) cells were transfected at ~80% confluence in 12-well dishes with 0.2 μg of plasmids encoding GFP or 0.5 μg of pc DNA3-Bcl-2 plus 0.2 μg of plasmids encoding GFP (control) or 0.1 μg of GFP-Bax, GFP-Bak, alone or in combination with 0.1, 0.2, or 0.5 μg of pcDNA3-Bcl-2. For apoptosis assays, floating and adherent cells were collected, fixed, and stained with 0.1 μg/ml DAPI. The percentages of apoptotic cells were determined by counting the GFP-positive cells displaying nuclear fragmentation and/or chromatin condensation (mean ± S.D.; n = 3).

plasmid was reduced (ranging from 0.1 to 0.5 μg), co-expression of Bcl-2 with Bax or Bak displayed impressive protection in HEK293T and HeLa cells in a dose-dependent manner (Fig. 4).

Assessment of Binding of Anti-apoptotic Bcl-2 Family Proteins to BH3 Peptides of Bax and Bak by Fluorescence Polarization Assays—The cell protection from Bax-induced cell death observed with anti-apoptotic Bcl-2 family proteins correlated

the findings from IP and GST pull-down experiments. However, unexpected outcomes occurred regarding the ability of Bcl-2, Bcl-W, and Bfl-1 to protect from Bak-mediated cell death, given that no binding of these proteins to Bak was observed in IP or GST pulldown experiments. To address this discrepancy, FPAs were carried out to elucidate the binding of anti-apoptotic Bcl-2 family proteins to Bax and Bak BH3 peptides.

FPAs were performed using recombinant purified anti-apoptotic Bcl-2 proteins and FITC-conjugated Bax or Bak BH3 peptides as previously reported (13, 16). Our FPA results showed that for Bax BH3 peptide, the rank order of binding affinities were Bcl-B > Bfl-1/Bcl-2/Bcl-X$_L$ > Mcl/Bcl-2. Bcl-B had an apparent EC$_{50}$ for Bax BH3 of 40 nM, whereas Mcl-1 and Bcl-2 showed only background binding, similar to GST alone. For Bak, the rank order of binding affinities was Bcl-X$_L$/Mcl-1 > Bfl-1/Bcl-2/Bcl-2 > Bcl-B. Bcl-X$_L$ and Mcl-1 bound the best, with apparent EC$_{50}$ of ~20 nM, followed by moderate binding of Bak to Bfl-1, Bcl-W, and Bcl-2, with affinities of ~100 nM. No binding of Bak BH3 peptide was observed for Bcl-B. GST control protein showed only background binding to Bax and Bak peptides in these assays (Fig. 5).

Unlabeled Bax and Bak BH3 peptides were used to compete with binding of FITC-Bax and FITC-Bak BH3 peptides to assess the specificity of the binding results obtained by FPA. Unlabeled Bax BH3 peptide competed effectively with FITC-Bax peptide for binding Bcl-B and unlabeled Bak BH3 peptide competed with FITC-Bak BH3 peptide for binding to Mcl-1 (Fig. 6). These data thus confirm competitive binding that was suppressed by unlabeled peptide to nearly the background level observed with FITC-Bax peptide alone (*i.e.* no Bcl-2 family proteins), with IC$_{50}$ values in the range of 1–10 μM.

We then extended these peptide competition studies, taking advantage of the ability of FITC-Bim BH3 peptide to bind all six anti-apoptotic Bcl-2 family proteins with high affinity (9). The inhibitory concentrations of unlabeled Bax and Bak required to suppress FITC-Bim-peptide binding by 50% (IC$_{50}$) were calculated for each protein (Table 1). In these assays where FITC-Bim binding to Bcl-B was measured, unlabeled Bax BH3 peptide effectively competed, whereas significant inhibition was not observed with unlabeled Bak BH3 peptide. Conversely, in FPAs with Mcl-1, binding of FITC-Bim was effectively suppressed with unlabeled Bak BH3 peptide, whereas unlabeled Bax BH3 peptide was >15-fold less effective (Table 1). Thus,
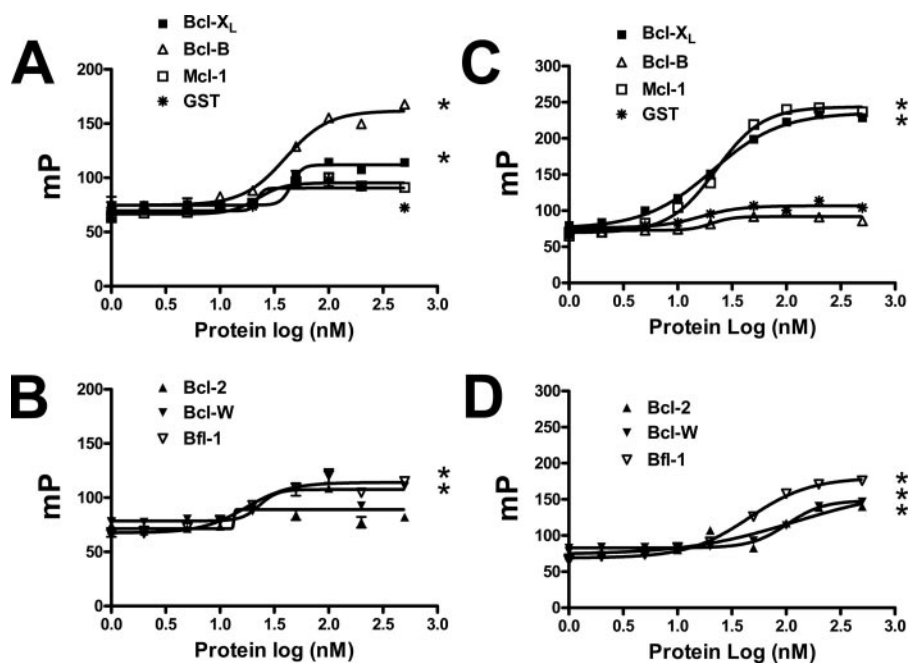
FIGURE 5. **FPA analysis of anti-apoptotic Bcl-2 family protein binding to Bak or Bax BH3 peptides.** Various concentrations of GST (control) or GST-Bcl-2 family proteins were incubated with 10 nM FITC-conjugated-Bax BH3 peptide (*A* and *B*) or 15 nM FITC-conjugated-Bak BH3 peptide (*C* and *D*). Fluorescence polarization was measured after 10 min. *mP*, millipolars. The concentration to achieve 50% maximal binding (EC$_{50}$) was calculated from the data. The data are shown in separate panels for clarity of presentation, because of overlapping curves. All of the data represent means ($n = 3$). Standard errors were ≤15% for all determinations. Statistical significance was determined by analysis of variance. The *asterisks* indicate p values < 0.01.
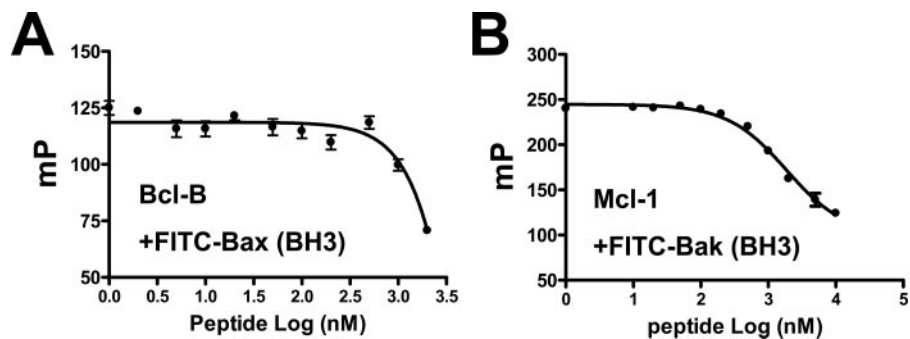
FIGURE 6. **FPA competition experiments.** Bcl-B (*A*) or Mcl-1 (*B*) (100 nM each) was incubated with unlabeled Bax or Bak BH3 peptide, respectively, for 2 min followed by the addition of 10 nM FITC-conjugated-Bax BH3 peptide (*A*) or 15 nM FITC-conjugated-Bak BH3 peptide (*B*). Fluorescence polarization was measured after 10 min. *mP*, millipolars. IC$_{50}$ values were calculated from the data (Bcl-B = 8.8 μM; Mcl-1 = 1.8 μM).

these peptide competition studies corroborate the direct FITC-BH3 peptide binding results and confirm that Bcl-B and Mcl-1 have strong preferences for Bax BH3 and Bak BH3, respectively. Interestingly, both unlabeled Bax and Bak BH3 peptides were effective in competing FITC-Bim peptide from Bcl-2 (Table 1), providing evidence that both Bax and Bak BH3 peptides are capable of competing for occupancy of the cleft on Bcl-2 where BH3 peptides bind. Although varying from the results obtained by direct binding of FITC-conjugated BH3 peptides, these FPA competition results are in agreement with the observation that Bcl-2 suppresses apoptosis induced by overexpression of either Bax or Bak.

DISCUSSION

Several studies have been performed regarding the relationship between BH3-only proteins and anti-apoptotic Bcl-2

proteins (8–10, 19). However, little is known regarding the mechanisms by which the anti-apoptotic Bcl-2 family proteins regulate the activity of the downstream multidomain family members, Bax and Bak. In this report, we examined the relationship between the heterotypic interactions of Bax and Bak with anti-apoptotic Bcl-2 family proteins and correlate the protein interaction data with their abilities to prevent Bax- and Bak-induced cell death. We observed that Bcl-B and Mcl-1 stand apart from the other anti-apoptotic human Bcl-2 family proteins in their differential binding to and suppression of the apoptotic activity of Bax and Bak, respectively. Unlike Bcl-2, Bcl-X$_L$, Bcl-W, and Bfl-1, which suppressed apoptosis induced by either Bax or Bak and which demonstrated physical interactions with both Bax and Bak by at least one of the three protein interaction methods employed, Bcl-B and Mcl-1 showed strong preferences for binding to and suppressing Bax and Bak, respectively.

IP and GST pulldown experiments are widely used to detect interactions between proteins, including the Bcl-2 family proteins. In our studies, we observed that Bax bound five of six anti-apoptotic Bcl-2 family proteins (all except Mcl-1) as assessed by IP and GST pulldown binding assays. Similarly, we found that Bcl-2, Bcl-X$_L$, Bcl-W, Bfl-1, and Bcl-B could all suppress Bax-induced apoptosis, whereas Mcl-1 did not. Thus, for Bax, binding data based on co-IP and GST pulldown correlated nicely with cell death induced by overexpression of Bax. In contrast, by FPA, no direct interaction was observed between the FITC-conjugated BH3 peptide of Bax and the Bcl-2 protein. However, in BH3 peptide competition experiments, unlabeled Bax BH3 peptide effectively competed with FITC-Bim BH3 peptide for binding to Bcl-2, suggesting that the BH3 peptide of Bax is competent to bind Bcl-2.

For Bak, five of the six Bcl-2 family members (all but Bcl-B) suppressed apoptosis induced by Bak overexpression, and these same five family members displayed interactions with the BH3 peptide of Bak by FPA. In contrast, only two of these anti-apoptotic Bcl-2 family proteins (Bcl-X$_L$; Mcl-1) demonstrated interactions with full-length protein by co-IP and GST pulldown assays. Thus, FPA data were more indicative of functional interactions for Bak than co-IP or GST pulldown. Note that our

TABLE 1

FPA data summary

FPA data are summarized for direct binding of FITC-BH3 peptides and for unlabeled BH3 peptide competition experiments. The concentrations of FITC-conjugated Bax (F-Bax) or Bak (F-Bak) BH3 peptide required to achieve 50% maximal binding (EC_{50}) are indicated, as measured by FPA (mean $\pm$ S.E.; $n = 3$). For competition experiments, Bcl-2 family proteins (100 nM) were incubated with various concentrations of unlabeled Bax or Bak peptides for 10 min, followed by 15 nM FITC-Bim BH3 peptide. The concentrations of Bax or Bak peptide required to inhibit FITC-Bim peptide binding by 50% (IC_{50}) are reported. The data represent averages of four determinations. The standard errors were $\leq$10%.

Protein	Binding EC_{50}		Competition IC_{50}	
	F-Bax	**F-Bak**	**Bax**	**Bak**
	nM		*μM*	
Bcl-X_L	45.4	20.9	13.2	1.5
Bcl-2	>1000	119.5	10.4	10.7
Bcl-W	24.7	101.9	7.8	2.8
Bcl-B	40.1	>1000	15.8	>50
Bfl-1	17.3	45.5	2.3	7.1
Mcl-1	>1000	22.5	32.9	1.8
GST	>1000	>1000		

IP and GST pulldown results concerning the interactions of Bcl-2 family proteins with Bak are nearly identical to those recently published (20) in that only Bcl-X_L and Mcl-1 displayed detectable associations with Bak. Thus, these results are independently verifiable. Interestingly, the cytoprotection observed with the three Bcl-2 family proteins that demonstrated interactions only by FPA (Bcl-2, Bfl-1, Bcl-W) was comparable with that observed with the two proteins that demonstrated strong binding to Bak in IP and GST pulldown assays (Bcl-X_L and Mcl-1), thus showing that FPA and cytoprotection data nicely correlate for Bak.

Several factors could contribute to the differential protein interaction results obtained by various methods (co-IP, GST pulldown, and FPA). Foremost among them is protein conformation. Bcl-2 family proteins are known to assume different conformational states that impact their ability to form homo- or hetero-dimers or oligomers in solution and in association with intracellular membranes. For example, the BH3 domains of Bax and Bak are known to mediate their physical interactions with anti-apoptotic Bcl-2 family proteins. The BH3 domains are comprised of amphipathic α-helices that bind primarily via their hydrophobic face to a hydrophobic pocket on anti-apoptotic Bcl-2 family proteins (21). To expose the hydrophobic surface of the BH3 peptide, Bax and Bak presumably must undergo profound conformational changes, given that the three-dimensional structure of the full-length Bax protein shows the BH3 domain rotated so that the hydrophobic surface is buried (22). In FPAs, the isolated BH3 peptide is used, thus obviating the influence of conformational differences in Bax or Bak for purposes of detecting potential interactions partners among the anti-apoptotic Bcl-2 family proteins. With co-IP and GST pulldown experiments, in contrast, full-length Bax and Bak proteins were used. Presumably at least some of the Bax and Bak expressed by transfection in cells is in the active conformation, given that these proteins displayed interactions with selected anti-apoptotic Bcl-2 family members. In this regard, we used CHAPS detergent for our protein interaction studies because of indications that it preserves the endogenous conformation of Bax. Thus, it should be considered that use of harsher detergents such as SDS might have forced more Bax and Bak

into active conformations and thereby might have revealed additional protein interactions not detected here.

At least for cell death induced by overexpression of Bak, FPA data correlate far better than co-IP or GST pulldown assays with the cellular function of anti-apoptotic Bcl-2 family proteins in terms of their ability to antagonize Bak. In the case of Bax-induced apoptosis, co-IP and GST pulldown data correlated well with functional antagonism by anti-apoptotic Bcl-2 family proteins. In fact, FPA data were less reliable indicators of function for Bax, in that recombinant Bcl-2 protein displayed little interaction with synthetic Bax BH3 peptide, yet Bcl-2 suppressed apoptosis induced by Bax overexpression. This observation implies that the mechanism by which Bcl-2 suppresses Bax in cells may differ from other anti-apoptotic Bcl-2 family proteins. Bcl-2, for example, could bind and titrate out upstream activators of Bax (such as Bid and Bim), or interact with Bax in membranes of mitochondria to prevent Bax oligomerization, yet not bind Bax BH3 peptides in solution. Although it is also possible that the GST fusion version of Bcl-2 used here for FPAs is artificially impaired in its ability to bind Bax BH3 peptides, this recombinant protein displays high affinity interactions with several other BH3 peptides, including Bid and Bim, with $EC_{50} \cong 10-80$ nM (Ref. 16 and data not shown). In this regard, the ability of some anti-apoptotic Bcl-2 family proteins to form dimers in solution could theoretically impact binding to BH3 peptides but still seems an inadequate explanation for differential binding of Bax and Bak. Of course, it is possible that the conformation of the synthetic Bax BH3 peptide differs from the conformation of the BH3 domain in the context of the full-length Bax protein, but the isolated Bax BH3 peptide nevertheless bound to several anti-apoptotic Bcl-2 family proteins with high affinity. Of note, although FITC-conjugated Bax BH3 peptide failed to bind Bcl-2, unconjugated Bax peptide effectively competed with another FITC-BH3 peptide (pig Bim) for binding to Bcl-2, thus implying that the addition of the FITC moiety may have uniquely impacted binding to Bcl-2 but not other members of the Bcl-2 family.

Altogether, the correlations of anti-apoptotic function with protein interaction data reported here argue that reliance on any one method for assessing interactions of Bax and Bak with anti-apoptotic Bcl-2 family proteins can be limiting and suggest that several methods should be compared before drawing firm conclusions. This therefore sets Bax and Bak apart from the BH3-only branch of the Bcl-2 family, where binding and function appear to be readily correlated by a variety of standard methods (reviewed in Refs. 23 and 24).

Bcl-2 stands apart from the other five anti-apoptotic human Bcl-2 family proteins in its propensity to induce apoptosis when overexpressed by transfection. As shown here, studies of the function of Bcl-2 as an anti-apoptotic protein required that we reduce plasmid concentrations by $\sim$10-fold relative to Bcl-X_L, Mcl-1, Bcl-W, Bfl-1, and Bcl-B to avoid apoptosis induction. Bcl-2 has been shown to display pro-apoptotic phenotypes under certain conditions. For example, upon interaction with the orphan nuclear receptor Nur77/TR3, Bcl-2 undergoes conformational changes that exposes its BH3 domain, making it analogous to pro-apoptotic BH3-only proteins (25). We presume that excessive overexpression of Bcl-2 favors pro-apopto-

tic conformations of this protein. It remains to be determined whether different conformational states of Bcl-2 impacted the interaction data obtained here for Bax and Bak full-length proteins and for Bax and Bak BH3 peptides, as measured by co-IP, GST pulldown, and FPA.

In summary, we show that Bax and Bak differ in their physical and functional interactions with the six human anti-apoptotic Bcl-2 family members. The greatest differences in cellular and biochemical behavior were seen for Bcl-B and Mcl-1. Bcl-B bound Bax but not Bak by all methods tested (co-IP, GST pull-down, and FPA), and Bcl-B suppressed apoptosis induced by overexpression of Bax but not Bak. Conversely, Mcl-1 bound Bak but not Bax by all methods tested, and Mcl-1 suppressed apoptosis induced by overexpression of Bak but not Bax. In contrast, setting aside quantitative differences in relative affinities, at a qualitative level, the other anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-X_L, Bcl-W, and Bfl-1) demonstrated interactions with both Bax and Bak by at least one method, and they all demonstrated an ability to suppress apoptosis induced by overexpression of either Bax or Bak. These findings thus suggest strong preferences of Bcl-B and Mcl-1 for Bax and Bak, respectively, and should help to stimulate further investigations of the relationships of the endogenous Bcl-B and Mcl-1 proteins in the context of cell death programs that are dependent on either Bax or Bak.

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