Biochem. J. (2003) **376**, 229–236 (Printed in Great Britain)

Characterization of the anti-apoptotic mechanism of Bcl-B

Dayong ZHAI*, Ning KE*, Haichao ZHANG†, Uri LADROR†, Mary JOSEPH†, Andreas EICHINGER*, Adam GODZIK*, Shi-Chung NG† and John C. REED*[1]

*The Burnham Institute, La Jolla, CA 92037, U.S.A., and †Cancer Research, Abbott Laboratories, Abbott Park, IL 60064, U.S.A.

Bcl-B protein is an anti-apoptotic member of the Bcl-2 family protein that contains all the four BH (Bcl-2 homology) domains (BH1, BH2, BH3 and BH4) and a predicted C-terminal transmembrane domain. Our previous results showed that Bcl-B binds Bax and suppresses apoptosis induced by over-expression of Bax; however, Bcl-B does not bind or suppress Bak. To explore the molecular basis for the differential binding and suppression of Bax and Bak, we studied the BH3 dimerization domains of Bax and Bak. Chimeric mutants of Bax and Bak were generated that swapped the BH3 domains of these pro-apoptotic proteins. Bcl-B associated with and blocked apoptosis induced by mutant Bak containing the BH3 domain of Bax, but not mutant Bax containing the BH3 domain of Bak. In contrast, Bcl-X_L protein bound and suppressed apoptosis induction by Bax, Bak and both BH3-domain chimeras. A strong correlation between binding and apoptosis suppression was also obtained using a series of alanine substitutions spanning the length of the Bax BH3 domain to identify critical residues for Bcl-B binding. Conversely, using structure-based modelling to design mutations in the BH3-binding pocket of Bcl-B, we produced two Bcl-B mutants (Leu^{86} → Ala and Arg^{96} → Gln) that failed to bind Bax and that also were unable to suppress apoptosis induced by Bax over-expression. In contrast, other Bcl-B mutants that still bound Bax retained protective activity against Bax-induced cell death, thus serving as a control. We conclude that, in contrast with some other anti-apoptotic Bcl-2-family proteins, a strong correlation exists for Bcl-B between binding to pro-apoptotic multidomain Bcl-2 family proteins and functional apoptosis suppression.

Key words: apoptosis, Bak, Bax, Bcl-2.

INTRODUCTION

Apoptosis is an evolutionarily conserved process that is critical for a wide variety of biological events, such as embryonic development, immunological defence, maintenance of tissue homoeostasis, and removal of non-instructed, misinstructed and damaged cells. Defects in apoptosis regulation contribute to many diseases, including cancer, autoimmunity, stroke, heart failure, neurodegeneration and AIDS [1].

Mitochondria constitute one of the centres of death control within cells. Mitochondria undergo profound changes in membrane integrity during the apoptosis process, leading to the release of apoptogenic proteins, including cytochrome *c*, AIF (apoptosis-inducing factor), SMAC/Diablo (second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI), Endo G (endonuclease G) and Omi/HtrA2 (high temperature requirement A2) [2,3]. The Bcl-2-family proteins represent a group of evolutionarily conserved proteins that govern the mitochondria-dependent cell-death pathway, functioning to either promote or suppress release of apoptosis-inducing proteins. Many Bcl-2 proteins are constitutively localized to the membrane of mitochondria, whereas others are induced to translocate to these organelles in response to specific stimuli [4–6].

Sequence homology within Bcl-2 family protein clusters into regions known as BH (Bcl-2 homology) domains, with various family members containing as many as four conserved BH domains, i.e. BH1, BH2, BH3 and BH4 [4–6]. All known anti-apoptotic members of the Bcl-2 family possess all four BH domains, and most also contain a C-terminal hydrophobic span of amino acids responsible for their insertion into membranes. In contrast, pro-apoptotic Bcl-2 family proteins are more diverse in their sequences, typically containing only a subset of the four recognized BH domains.

Members of the Bcl-2 family form homo- and hetero-dimers under certain conditions. Many experiments have suggested that dimerization of pro- and anti-apoptotic Bcl-2-family proteins plays an important role in controlling their activity [6,7]. The BH3 domain of pro-apoptotic Bcl-2-family proteins constitutes an amphipathic α-helix that is sufficient for inducing apoptosis and for dimerization with anti-apoptotic family proteins [5,8,9]. Two classes of BH3-containing pro-apoptotic Bcl-2-family proteins have been delineated, and are known as ‘BH3-only’ versus ‘multidomain’ proteins. For the structurally diverse BH3-only proteins, an excellent correlation exists between BH3-domain-mediated dimerization with anti-apoptotic Bcl-2 family proteins and induction of apoptosis. In contrast, for the multidomain pro-apoptotic proteins (which typically contain BH1, BH2 and BH3 domains) such as Bax and Bak, BH3-mediated dimerization does not uniformly correlate with their apoptosis-inducing activity [10,11]. Similarly, for the anti-apoptotic Bcl-2-family proteins such as Bcl-2 and Bcl-X_L, the ability to dimerize with pro-apoptotic family members does not always correlate with their cytoprotective function [10,11].

Six anti-apoptotic Bcl-2 family proteins have been identified in humans and mice, excluding splicing variants and isoforms arising through gene duplication (reviewed in [12]). The most recently discovered anti-apoptotic member of the human Bcl-2 family is Bcl-B (also known as Nrh) [13–15]. The closest homologue to Bcl-B in mice is Diva/Boo, which is expressed exclusively in the ovary and which has been reported to possess either anti- or pro-apoptotic activity, depending on cellular context [16,17]. Unlike Diva/Boo of mice, however, the human Bcl-B protein is

Abbreviations used: Bcl-B(ΔTM), Bcl-B lacking its C-terminal transmembrane domain; BH, Bcl-2 homology; DAPI, 4,6-diamidino-2-phenylindole; DEVD-AFC, Asp-Glu-Val-Asp 7-amino-4-trifluoromethylcoumarin; DTT, dithiothreitol; FPA, fluorescence polarization assay; GFP, green fluorescent protein; L63A etc, Leu^{63} → Ala etc; KX, Bak chimera containing the BH3 domain from Bax; NP40, Nonidet P40; XK, Bax chimera containing the BH3 domain of Bak.

[1] To whom correspondence should be addressed (e-mail jreed@burnham.org or reedoffice@burnham.org).

© 2003 Biochemical Society

widely expressed in tissues of humans. Previous studies of Bcl-B indicated that this protein selectively binds the multidomain pro-apoptotic protein Bax, but not Bak, and that it suppresses apoptosis induced by over-expression of Bax, but not Bak [13]. In the present paper, we used mutagenesis approaches to explore further the anti-apoptotic mechanism of the Bcl-B protein. Our findings reveal a strong correlation between the ability of Bcl-B to bind multidomain pro-apoptotic Bcl-2 family proteins and its anti-apoptotic function.

EXPERIMENTAL

Plasmid construction

BH3-domain swap mutants of Bax with Bak were created in which the BH3 domains of these proteins were swapped, creating chimeric proteins. Specifically, a 21-amino-acid segment of Bax, Q^{52}DASTKKLSECLKRIGDELDS^{72}, was swapped with the P^{67}S-STMGQVGRQLAIIGDDINR^{87} region of Bak. To accomplish this, two primers (forward primer 5′-TGTCTCAAGCGCATC-GGGGACGAACTGGACAGTCGCTATGACT CAGAGTTCC-AA-3′, and reverse primer, 5′-CTCGCTCAGCTTCTTGGTGG-ACGCATCCTGTTGCAGAGGTAAGGTGACCATC-3′) were employed with a Bax cDNA template (pEGFP-Bax) [10] using a one-step PCR-based mutagenesis kit (Stratagene; Quik-Change). Alternatively, mutagenic primers (forward primer, 5′-CTCGCCATCATCGGGGACGACATCAACCGAAACATGG-AGCTGCAGAGGATGATT-3′, and reverse primer, 5′-CTGCCG-TCCCACCTGCCCCATGGTGCTGCTAGGAGGCACCGGG-TCCAGGGCCA GCT-3′) were employed with a Bak cDNA template (pEGFP-Bak) [18]. Each pair of the primers contains both the Bax or Bak sequence upstream or downstream of their BH3 domains and the sequence of the swapped BH3 domain of Bak or Bax. Pfu polymerase was used to reduce the poly-adenylated tailing of PCR products which were cloned into pEGFP-Bax or pEGFP-Bak by blunt-end ligation.

Site-directed mutagenesis of Bax was performed to generate the L63A ($Leu^{63} \rightarrow$ Ala), K64A, D68A, E69A and L70A mutants, and site-directed mutagenesis of Bcl-B was performed to generate the substitution mutants L86A, R96Q and a mutant of Bcl-B in which three alanines were substituted for the sequence $F^{169}FR^{171}$, using the QuikChange™ site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol, with pcDNA3-HA-Bax and pcDNA3-Myc-Bcl-B plasmid as DNA templates respectively [13,19]. Two mutagenic primers were used to generate the Bcl-B(Δ118–133) loop deletion mutant by a similar method: 5′GGGCCGCTGGTGACCGCCCGGGTCG-CCCGGGACTGCCAGCGC-3′ and 5′-GCGCTGGCAGTCCC-GGGCGACCCGGGCGGTCACCAGCGGCCC-3′. A cDNA encoding Bcl-B lacking its C-terminal transmembrane domain [Bcl-B(ΔTM)] was constructed by engineering a termination codon after residue Val^{184} of the protein and cloned into the *Eco*RI and *Xho*I sites of the bacterial expression vector pGEX 4T-1.

Protein purification

The GST (glutathione S-transferase)–Bcl-B fusion protein was expressed from pGEX 4T-1-Bcl-B (ΔTM) in XL-1 Blue cells (Stratagene). Briefly, cells were grown in 2 litres of Luria–Bertani broth with 50 μg/ml ampicillin at 37 °C to an D_{600} of 1.0, then 0.5 mM IPTG (isopropyl β-D-thiogalactoside) was added, and the cultures were incubated at 25 °C for 6 h. Cells were then recovered and incubated with 0.5 mg/ml lysozyme in 20 mM phosphate buffer (pH 7.4), 150 mM NaCl, 1 mM DTT (dithiothreitol), 1 mM EDTA and 1 mM PMSF at room temperature for 10 min, followed by sonication. Cellular debris was pelleted by centrifugation at 27 500 ***g*** for 20 min, and the resulting supernatants were incubated with 10 ml of glutathionine–Sepharose (Pharmacia) at 4 °C for 2 h. The resin was washed 3 times with 20 mM phosphate buffer (pH 7.4), 150 mM NaCl and 1 mM DTT, and then 10 mM of reduced glutathione dissolved in 50 mM Tris/HCl (pH 8.0) was used to elute the GST–Bcl-B protein. Further purification was achieved by anion-exchange chromatography, using a Mono S column (Pharmacia). The resulting GST–Bcl-B protein was dialysed with 50 mM Tris/HCl (pH 7.5)/1 mM DTT. The purity of GST–Bcl-B protein was > 95 % as determined by Coomassie Blue staining of the SDS/PAGE gels.

Fluorescence polarization assays (FPAs)

To determine the binding affinity of different BH3 peptides to Bcl-B protein, FPAs were performed as described previously [20]. Briefly, various concentrations of GST–Bcl-B fusion protein were incubated with 5 nM of FITC-conjugated synthetic purified Bax, Bak or Bad BH3 domain peptides dissolved in DMSO in the dark. Fluorescence polarization was measured using an Analyst™ AD Assay Detection System (LJL Biosystem, Sunnyvale, CA, U.S.A.). IC_{50} determinations were performed as described previously [21], using a GraphPad Prism software package. To determine the contribution of individual amino acid residues of Bax BH3 peptide to the binding to Bcl-B, a series of mutant Bax BH3 peptides with alanine substitutions were used in competitive inhibition FPA. In this assay format, the unlabelled peptides were used to compete against the fluorescein-labelled Bax BH3 peptide (5 nM) for the binding to Bcl-B protein (120 nM). The IC_{50}s were derived from comparison with the relative affinities.

Cell culture, transfection and apoptosis assays

HEK-293T and COS7 cells were maintained in Dulbecco's modified Eagle's medium (Irvine Scientific) supplemented with 10 % fetal bovine serum, 1 mM L-glutamine and antibiotics (1 % penicillin/1 % streptomycin). For transient transfection apoptosis assays, cells (5×10^5) in six-well dishes were co-transfected using LIPOFECTAMINE Plus™ (Invitrogen) with 0.5 μg of pEGFP-C1 (ClonTech), pEGFP-Bax, pEGFP-Bax(BH3-K) (swap mutant of Bax containing the BH3 domain of Bak), pEGFP-Bak or pEGFP-Bak(BH3-X) (swap mutant of Bak containing the BH3 domain of Bax) plasmids together with 1.5 μg of pcDNA3-myc-Bcl-B or pcDNA3-myc-Bcl-X_L plasmids. Both floating and adherent cells were collected, fixed and stained with 0.1 mg/ml 4,6-diamidino-2-phenylindole (DAPI). The percentages of apoptotic cells were determined by counting the GFP (green fluorescent protein)-positive cells having nuclear fragmentation and/or chromatin condensation (means ± S.D.; $n = 3$). Statistical significance of apoptosis data was determined by unpaired Student's *t* test, with $P \leqslant 0.01$ as the threshold for significance.

Caspase activity assays

HEK-293T cells were co-transfected with 0.5 μg of control plasmid or pcDNA3-Bax together with 2 μg of plasmids encoding Bcl-X_L, Bcl-B or various mutants of Bcl-B. Cell lysates were prepared after 24 h, normalized for protein content and 25 μg of

© 2003 Biochemical Society

cell lysate was incubated with 100 μM DEVD-AFC (Asp-Glu-Val-Asp 7-amino-4-trifluoromethylcoumarin), measuring enzyme activity by the release of AFC (7-amino-4-trifluoromethylcoumarin) fluorescence as described previously [22]. Statistical significance of results was determined by unpaired Student's *t* test, with $P \leqslant 0.01$ as the threshold for significance.

Co-immunoprecipitation and immunoblotting assays

Immunoblotting was performed as described previously [13]. Transfected HEK-293T cells (5×10^5) were cultured with 50 μM benzoyl-Val-Ala-Asp-fluoromethylketone (Bachem) to prevent cell death. At 24 h post-transfection, cells were collected and resuspended in lysis buffer [10 mM Hepes (pH 7.4), 142.4 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.2 % NP40 (Nonidet P40) or 2 % CHAPS] containing 12.5 mM β-glycerophosphate, 2 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF and a protease inhibitor mixture (Roche Molecular Biochemicals). Soluble lysates were incubated with 10 μl of anti-Myc (Santa Cruz) antibody-conjugated Sepharose beads overnight at 4 °C. Beads were then washed four times in 1 ml of lysis buffer and boiled in an equal volume of Laemmli gel-loading solution before analysis of proteins by SDS/PAGE and/or immunoblotting using polyclonal rabbit anti-GFP (Roche Molecular Biochemicals) or monoclonal mouse anti-Myc (Santa Cruz) antibodies.

Molecular modelling

Modelling of the Bcl-B interactions with Bax and Bak was accomplished by a three-step procedure. First, a full three-dimensional model of Bcl-B was built on to a Bcl-2 template (Protein DataBank accession number 1G5M), based on FFAS [23] alignments and using the MODELLER program [24]. In addition, the BH3 domains of Bax and Bak were modelled separately, using the same procedure as described above. In a second step, possible structures of Bcl-B–BH3 domain complexes from Bax or Bak were built using a docking program GRAMM [25]. Finally, all complexes were evaluated using detailed energy calculations and careful residue level analysis. Models of several different substitution mutants were created by substituting appropriate residues in the model of the complex and repeating the energy minimization analysis.

RESULTS

The BH3 domains of Bax and Bak dictate their ability to bind Bcl-B

Our previous studies showed that the Bcl-B protein binds and suppresses apoptosis induced by Bax but not Bak [13]. Since anti-apoptotic Bcl-2-family proteins contain a hydrophobic crevice that binds BH3 peptides from pro-apoptotic family members [9], we tested the hypothesis that differences in the BH3 domains of Bax and Bak account for their differential binding to Bcl-B. Accordingly, we constructed expression plasmids encoding chimeras of Bax and Bak in which their BH3 domains were swapped. Co-immunoprecipitation assays were then used to contrast the ability of the wild-type and chimeric proteins to bind Bcl-B, upon transient transfection in HEK-293T cells. For comparative purposes, identical experiments were performed using Bcl-X_L instead of Bcl-B. For these experiments, Bcl-B and Bcl-X_L were expressed with N-terminal Myc-epitope tags, whereas Bax, Bak and the chimeric mutants were expressed with N-terminal GFP tags.

As shown in Figure 1, Bcl-B interacted with Bax, and the Bak chimera containing the BH3 domain from Bax (abbreviated as KX), but not Bak and not the Bax chimera containing the BH3 domain of Bak (abbreviated as XK). Similar results were obtained regardless of whether lysis solution contained NP40 (Figures 1A and 1B) or CHAPS (Figure 1C), a detergent that is thought to more closely reflect the physiological interactions of Bcl-2-family proteins [26]. In contrast, Bcl-X_L demonstrated binding to all of these proteins, but not to control GFP protein. Immunoblot analysis confirmed production of Bcl-B, Bcl-X_L, Bax, Bak and chimeric proteins at comparable levels, excluding trivial explanations for the failure of Bcl-B to interact with Bak and XK. The Myc-tagged Bcl-B expressed in HEK-293T cells also displayed selective interactions with the endogenous Bax protein, but not the endogenous Bak protein in co-immunoprecipitation assays, whereas Bcl-X_L interacted with both endogenous Bax and Bak (Figure 1D).

The BH3 domains of Bax and Bak determine sensitivity to Bcl-B-mediated apoptosis suppression

To correlate binding with function, we tested the ability of Bcl-B to suppress apoptosis induced by Bax, Bak and the XK and KX chimeric proteins. Again, comparisons were made with Bcl-X_L. When plasmids encoding Bax, Bak, XK or KX were transiently transfected into HEK-293T (Figure 2A) or COS7 (Figure 2B) cells, approx. 80 % of the transfected cells (marked by GFP) underwent apoptosis within 24 h, as determined by DAPI staining. Co-expression of Bcl-B markedly suppressed apoptosis induced by Bax or KX, but not by Bak or XK, in both HEK-293T and COS7 cells. In contrast, Bcl-X_L was effective at suppressing apoptosis induced by all these proteins (Figure 2). As shown above, the failure of Bcl-B to suppress apoptosis induced by Bak and XK was not due to differences in protein production. We conclude therefore that the BH3 domains of Bax and Bak determine whether these proteins are sensitive to apoptosis suppression by Bcl-B.

Bcl-B protein binds to BH3 peptide of Bax but not Bak, Bid or Bad

FPAs were used to directly contrast the binding of Bcl-B to the BH3 peptides of several pro-apoptotic Bcl-2 family proteins. For these experiments, we purified recombinant GST–Bcl-B(ΔTM) protein and employed it in combination with FITC-labelled synthetic peptides corresponding to the sequences of the BH3 domains of Bax, Bak and Bad (Figure 3A). For optimal results, FITC was conjugated at different sites within the BH3 peptides, empirically optimizing the conjugation site based on binding to recombinant Bcl-X_L(ΔTM) protein (results not shown). For the Bak and Bad BH3 peptides, this required substituting lysine residues to provide a primary amino group for FITC conjugation (i.e. Bak D84K and Bad E120K).

Among the BH3 peptides tested, Bcl-B bound only to the Bax peptide, with an IC_{50} of approx. 175 nM (Figure 3B). No binding was detected for the FITC-labelled Bad, Bid and Bak peptides up to 1 μM (Figure 3B and results not shown). To exclude artifacts in the binding assay attributable to the conjugation of FITC to BH3 peptides, we also performed competition experiments in which unlabelled wild-type Bax, Bak, Bad,and Bid BH3 peptides were tested for ability to compete with FITC-labelled Bax peptide for binding to Bcl-B. For the competition experiments, we used 5 nM FITC–Bax and 120 nM GST–Bcl-B(ΔTM), which was empirically determined to provide an optimal signal-to-noise

© 2003 Biochemical Society

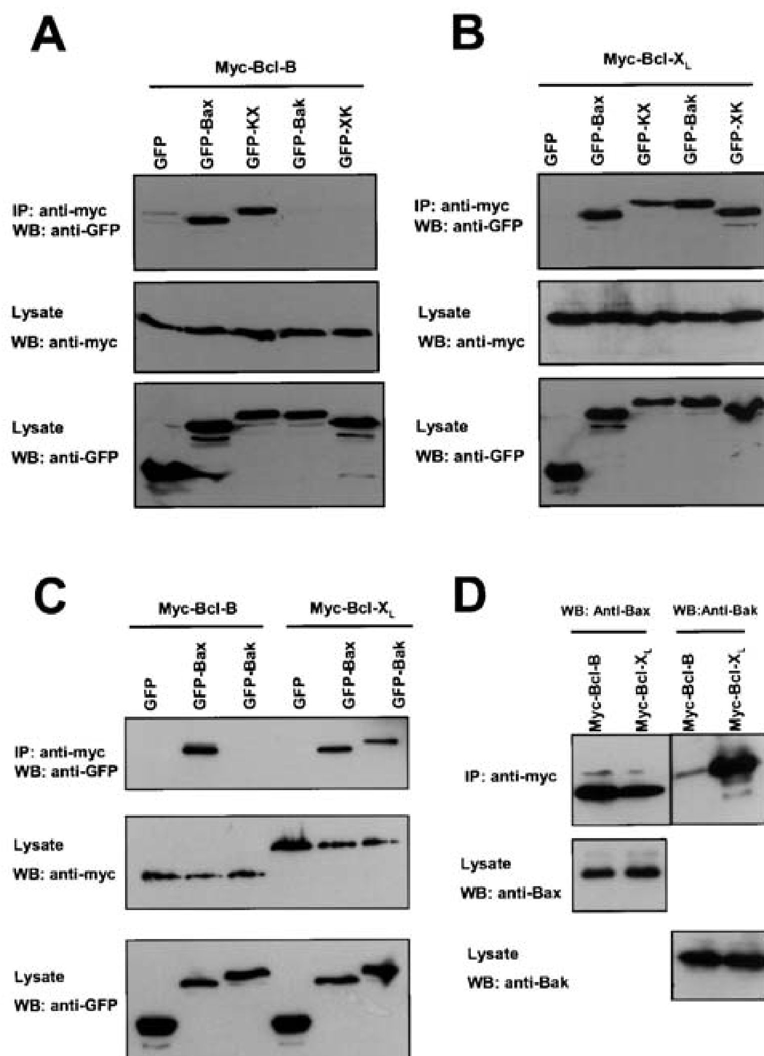

Figure 1 Analysis of binding of Bcl-B and Bcl-X_L to BH3 domain chimeras of Bax and Bak

(**A**–**C**) HEK-293T cells in 100 mm-diameter dishes were transiently transfected with 0.5 μg of plasmids encoding GFP–Bax, GFP–Bak, GFP–Bax containing the BH3 domain of Bak (XK), GFP–Bak containing the BH3 domain of Bax (KX) or 0.5 μg of GFP control, together with 1 μg of pcDNA3-Bcl-B or pcDNA3–Bcl-X_L. Cells were cultured for 24 h in 50 μM benzoyl-Val-Ala-Asp-fluoromethylketone to avoid apoptosis, then lysed in solutions containing either 0.2 % NP40 (**A** and **B**) or 2 % CHAPS (**C**) detergent. Lysates were normalized for total protein concentration, and then subjected to immunoprecipitation, employing anti-Myc antibody. Immune complexes were analysed by SDS/PAGE and immunoblotting, using mouse monoclonal anti-GFP antibody (top panel). To verify expression of all proteins, equivalent volumes of lysates were also loaded directly on to gels and analysed by SDS/PAGE and immunoblotting using antibodies against Myc (middle panel) or GFP (bottom panel). (**D**) HEK-293T cells were transfected with 0.5 μg of pcDNA3-Bcl-B or pcDNA3-Bcl-X_L without Bax- or Bak-encoding plasmids. NP40-containing lysates were subjected to immunoprecipitation using anti-Bax (33) or anti-Bak (31) sera, and the resulting immune complexes were analysed by SDS/PAGE and immunoblotting using anti-Bax or anti-Bak antibodies (top panel), anti-Bax (middle panel) or anti-Bak (bottom panel). For efficiency of presentation, only the portion of gels containing the relevant bands is shown. IP, immunoprecipitation; WB, Western blotting.

ratio. As shown in Figure 3(C), unlabelled Bax peptide effectively competed with the FITC–Bax peptide for binding to Bcl-B, whereas the unlabelled wild-type Bak, Bad and Bid peptides did not. Thus we conclude that Bcl-B binds selectively to the BH3 domain of Bax.

Identification of critical residues in Bax BH3 domain required for binding to Bcl-B

The residues within the Bax BH3 peptide required for binding to Bcl-B were defined by alanine substitutions spanning the entire length of the Bax BH3 peptide (Figure 4). Since alanine naturally occupies position number 3 of the Bax BH3 peptide, we substituted a glycine at this position. Again, 5 nM FITC–Bax wild-type peptide and 120 nM GST–Bcl-B(ΔTM) protein were used for these competition experiments, and IC_{50} values were calculated for each peptide. These experiments showed that Thr^{56}, Leu^{59}, Glu^{61}, Leu^{63}, Lys^{64}, Ile^{66} and Leu^{70} make important contributions to binding of Bax BH3 peptides to Bcl-B, increasing the IC_{50} by approx. 20-fold or more relative to wild-type Bax BH3 peptide, which displayed an IC_{50} of approx. 0.5 μM under the conditions of these assays (Figure 4).

Use of Bax mutants for correlating binding and apoptosis suppression by Bcl-B

Previously, we generated and characterized mutants of the Bax protein in which specific residues within the BH3 domain were

© 2003 Biochemical Society

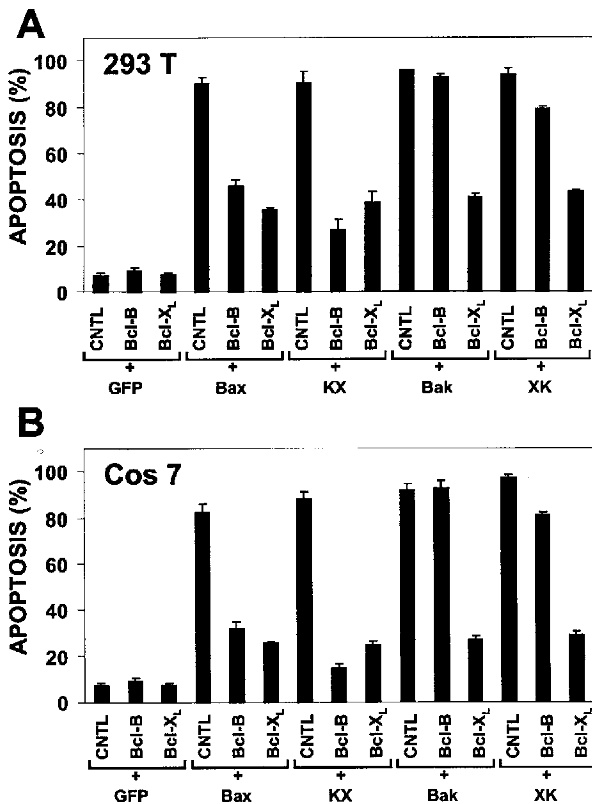

Figure 2 Bcl-B only inhibits apoptosis induced by the Bax/Bak chimeras that it can bind

HEK-293T (**A**) or COS7 (**B**) cells at approx. 50 % confluence in six-well dishes were co-transfected with 0.5 μg of plasmids encoding GFP (control) or GFP–Bax, GFP–Bak, GFP–XK, or GFP–KX alone or in combination with 1.5 μg of pcDNA3-Bcl-B or pcDNA3-Bcl-X_L. After 24 h, cells were collected, fixed and stained with DAPI. The percentage of GFP-positive cells with apoptotic morphology (fragmented nuclei or condensed chromatin) was determined (means $\pm$ S.D.; $n = 3$). Data are representative of several experiments. CNTL, control.

mutated, including L63E, D68A and E69A [10]. Based on the FPA data presented above, we expected the L63A, K64A and L70A mutants to display markedly reduced binding to Bcl-B, whereas the D68A and E69A mutations should not be deleterious to Bcl-B binding. Taking advantage of the availability of expression plasmids encoding these mutant Bax proteins, we compared the ability of Bcl-B to associate with them in cells by co-immunoprecipitation assays (Figure 5A) and to suppress apoptosis in cells over-expressing these Bax mutants (Figure 5B). Bcl-B retained the ability to bind the D68A and E69A mutants of Bax, and inhibited apoptosis when over-expressed in HEK-293T cells. In contrast, Bcl-B failed to bind the L63A, K64A and L70A mutants of Bax, and also did not suppress apoptosis induced by these proteins (Figure 5). Thus a correlation exists for Bcl-B between binding to Bax and suppressing Bax-induced apoptosis.

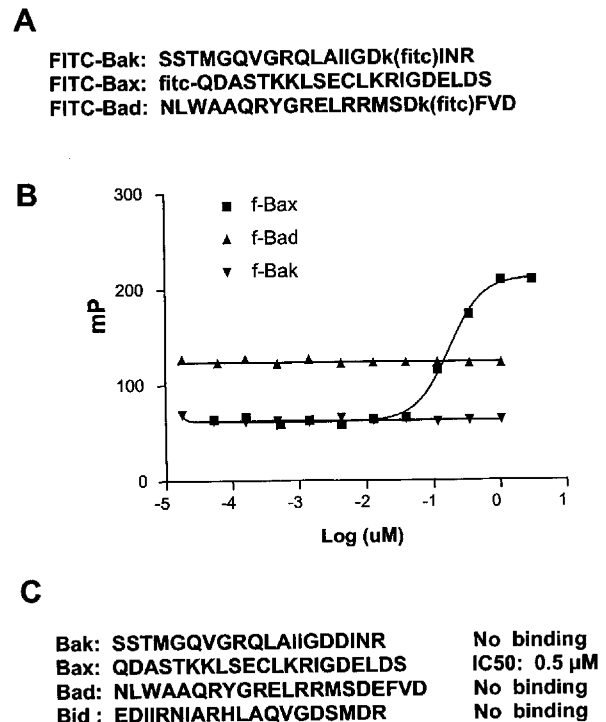

Figure 3 FPA analysis of Bcl-B binding to BH3 peptides

FPAs were used to study the specificity of Bcl-B for various BH3 peptides. (**A**) The sequences of the BH3 peptides used for FPA studies are presented, indicating the site at which the FITC fluorochrome was conjugated (fitc). Lysines were substituted for the endogenous sequences in those cases where FITC was placed internally within the peptide. (**B**) Various concentrations of GST–Bcl-B(ΔTM)-fusion protein were incubated in the dark with 5 nM of FITC-conjugated synthetic Bax, Bak or Bad BH3 peptides dissolved in DMSO. Fluorescence polarization was measured (mP). (**C**) Competition studies were performed using various concentrations of unlabelled BH3 peptides as indicated, added to 5 nM FITC–Bax and 120 nM GST–Bcl-B(ΔTM), measuring polarization (mP) after 15 min.

52																				72	µM
Q	D	A	S	T	K	K	L	S	E	C	L	K	R	I	G	D	E	L	D	S	0.5
Q	D	**G**	S	T	K	K	L	S	E	C	L	K	R	I	G	D	E	L	D	S	6.9
Q	D	A	**A**	T	K	K	L	S	E	C	L	K	R	I	G	D	E	L	D	S	1.9
Q	D	A	S	**A**	K	K	L	S	E	C	L	K	R	I	G	D	E	L	D	S	9.9
Q	D	A	S	T	**A**	K	L	S	E	C	L	K	R	I	G	D	E	L	D	S	1.1
Q	D	A	S	T	K	**A**	L	S	E	C	L	K	R	I	G	D	E	L	D	S	1.0
Q	D	A	S	T	K	K	**A**	S	E	C	L	K	R	I	G	D	E	L	D	S	15.3
Q	D	A	S	T	K	K	L	**A**	E	C	L	K	R	I	G	D	E	L	D	S	1.2
Q	D	A	S	T	K	K	L	S	**A**	C	L	K	R	I	G	D	E	L	D	S	15.1
Q	D	A	S	T	K	K	L	S	E	**A**	L	K	R	I	G	D	E	L	D	S	3.3
Q	D	A	S	T	K	K	L	S	E	C	**A**	K	R	I	G	D	E	L	D	S	18.1
Q	D	A	S	T	K	K	L	S	E	C	L	**A**	R	I	G	D	E	L	D	S	19.8
Q	D	A	S	T	K	K	L	S	E	C	L	K	**A**	I	G	D	E	L	D	S	5.5
Q	D	A	S	T	K	K	L	S	E	C	L	K	R	**A**	G	D	E	L	D	S	10.3
Q	D	A	S	T	K	K	L	S	E	C	L	K	R	I	**A**	D	E	L	D	S	0.6
Q	D	A	S	T	K	K	L	S	E	C	L	K	R	I	G	**A**	E	L	D	S	8.6
Q	D	A	S	T	K	K	L	S	E	C	L	K	R	I	G	D	**A**	L	D	S	4.3
Q	D	A	S	T	K	K	L	S	E	C	L	K	R	I	G	D	E	**A**	D	S	23.0
Q	D	A	S	T	K	K	L	S	E	C	L	K	R	I	G	D	E	L	**A**	S	4.1
Q	D	A	S	T	K	K	L	S	E	C	L	K	R	I	G	D	E	L	D	**A**	0.5
			*				*		*		*	*		*				*			

Figure 4 Alanine scanning replacement analysis of Bax BH3 peptide binding to Bcl-B

A series of alanine substitutions spanning the length of the Bax BH3 peptide were used at various concentrations in a competition assay with 5 nM FITC–Bax and 120 nM GST–Bcl-B(ΔTM). IC_{50} determinations were performed for each peptide and are shown on the right. Asterisks at bottom indicate those residues that resulted in a ⩾19-fold increase in IC_{50}.

Mutants of Bcl-B that fail to bind Bax also lack cytoprotective activity

To further explore the relation of Bax binding to anti-apoptotic function of Bcl-B, we used structure-based modelling to design mutations in the BH3-binding pocket of Bcl-B in an effort to create versions of Bcl-B that fail to bind Bax. For this purpose, Bcl-B was modelled on the reported structures of Bcl-X_L and Bcl-2 [27–29], with BH3 peptides from Bak and Bax respectively docked into the structures. Additional mutants of Bcl-B were also made in other regions of the protein during the course of these

© 2003 Biochemical Society

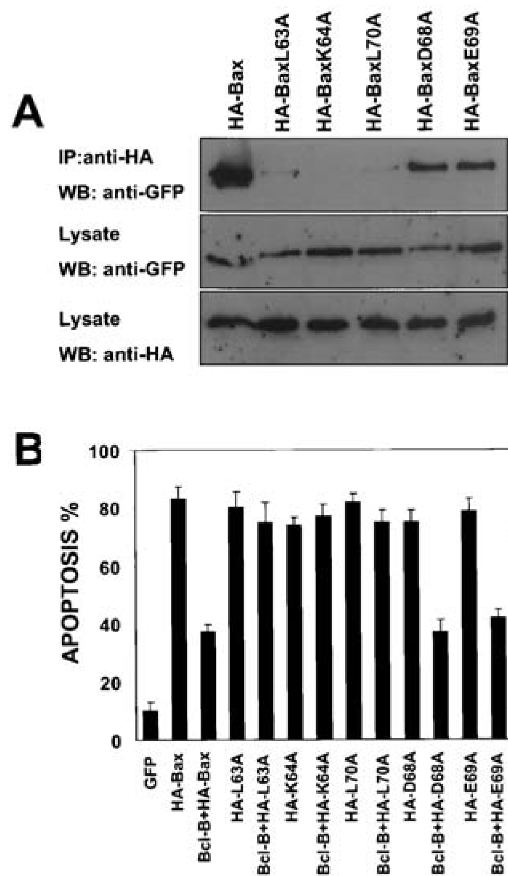

Figure 5 Bcl-B fails to block apoptosis induced by Bax mutants that it cannot bind

The ability of Bcl-B to bind and block apoptosis induced by various Bax mutants was tested. HEK-293T cells were transiently transfected with plasmids encoding HA (haemagglutinin)–Bax, or HA–Bax with the following substitutions, L63A, K64A, L70A, D68A or E69A, with pcDNA3-HA control or pEGFP-Bcl-B. (**A**) Lysates were prepared from transfected cells after 24 h using NP40-lysis buffer, and the lysates were used for immunoprecipitation assays employing anti-HA antibody. Immune complexes were analysed by SDS/PAGE and immunoblotting using anti-GFP antibody. (**B**) After 24 h, cells were collected, fixed and stained with DAPI. The percentage of GFP-positive cells with apoptotic morphology (fragmented nuclei or condensed chromatin) was determined (means ± S.D.; $n = 3$). Data are representative of several experiments.

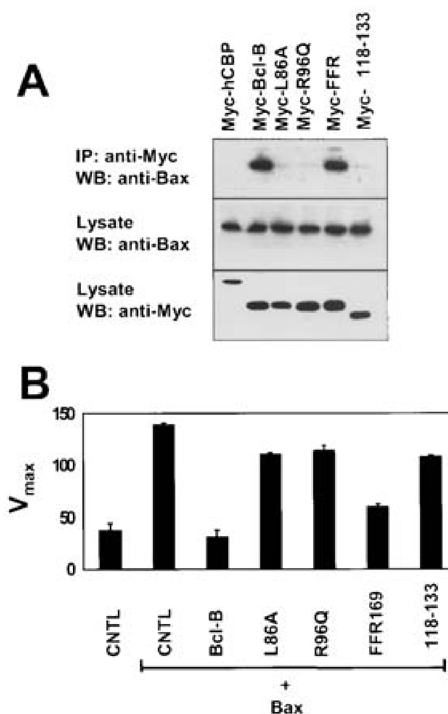

Figure 6 Bcl-B mutants that fail to bind Bax are defective in blocking Bax-induced caspase activation

(**A**) HEK-293T cells were transiently transfected with various plasmids encoding Myc-tagged proteins, including hCBP (human CREB-binding protein, used as a control) (Myc-hCBP), wild-type Bcl-B (Myc-Bcl-B), Bcl-B(L86A) (Myc-L86A), Bcl-B(R96Q) (Myc-R96Q), mutant of Bcl-B in which three alanines were substituted for the sequence $F^{169}FR^{171}$ (Myc-FFR), or Bcl-B(Δ118–133) (Myc-Δ118–133). Cell lysates were prepared and immunoprecipitated with anti-Myc antibody conjugated beads. Immunoprecipitates were then subject to SDS/PAGE and immunoblot analysis using rabbit polyclonal antibody specifically against Bax. To verify expression of proteins, lysates were also normalized for total protein content (25 μg), and subjected to immunoblot analysis using antibodies specifically against either Bax (middle panel) and or Myc (bottom panel). (**B**) For caspase assays, HEK-293T cells were co-transfected with 0.5 μg of Bax and 1.0 μg of various Bcl-B-encoding plasmids. Cell lysates were prepared 24 h after transfection, normalized for protein content (25 μg), and incubated with 100 μM DEVD-AFC. Enzyme activity was determined by the release of AFC fluorescence, and V_{max} was calculated (means ± S.D.; $n = 3$). CNTL, control; L86A, Bcl-B(L86A); R96Q, Bcl-B(R96Q); FFR169, mutant of Bcl-B in which three alanines were substituted for the sequence $F^{169}FR^{171}$; Δ118–133, Bcl-B(Δ118–133).

experiments, and used to test the correlations between binding to Bax and suppression of Bax-induced activation of caspases.

Two mutations in the BH3-binding pocket of Bcl-B, L86A and R96Q, ablated Bax binding activity, as demonstrated by co-immunoprecipitation assays using lysates from transiently transfected HEK-293T cells (Figure 6A). In addition, deletion of a loop between the first and second α-helices of Bcl-B(Δ118–133) also abolished Bax binding. These mutants of Bcl-B that lacked Bax-binding activity also failed to suppress caspase activation induced by over-expression of Bax (Figure 6B). In contrast, a mutant of Bcl-B in which three alanines were substituted for the sequence $F^{169}FR^{171}$ within the predicted BH3-binding pocket retained the ability to bind Bax, and also suppressed caspase activation induced by over-expression of Bax (Figure 6).

These data derived from mutants of Bcl-B thus provide further evidence that suppression of Bax-induced apoptosis by Bcl-B correlates with its ability to dimerize with Bax. We presume that the L86A and R96Q mutations in the BH3-binding pocket of Bcl-B directly interfere with binding to the BH3 domain of Bax, whereas the loop-deletion mutant presumably distorts the geometry of the BH3-binding pocket or otherwise disturbs Bax binding through a more indirect mechanism.

DISCUSSION

Bcl-2 family proteins differ in their preferences for dimerization partners, constituting a complex network of homo- and hetero-dimers. Resolving the roles of specific interactions among Bcl-2 family proteins is important for understanding the biology of these proteins and for devising potential therapeutic strategies. Previously, we reported that Bcl-B can differently bind and regulate the activity of Bax and Bak, but the relevant mechanism was unclear [13]. We show here that the BH3 domain represents the critical region of the Bax protein required for its association with and sensitivity to suppression by Bcl-B. Swapping the BH3 domain of Bax into the Bak protein converted Bak into a Bcl-B-binding protein, correlating with sensitivity to Bcl-B-mediated apoptosis suppression. Conversely, substituting the BH3 domain

© 2003 Biochemical Society

of Bak into the Bax protein abolished its ability to bind Bcl-B and rendered the protein resistant to apoptosis suppression by Bcl-B. Thus the BH3 domain of Bax dictates its sensitivity to suppression by Bcl-B. This correlation between binding and function was further supported by mutagenesis studies in which mutants of Bax that lost Bcl-B-binding activity were found to be resistant to Bcl-B with respect to cell death. Similarly, mutants of Bcl-B that lost the ability to bind Bax were inactive at suppressing Bax-induced cell death.

These findings have important implications for understanding the biological roles of Bcl-B, in as much as Bcl-B would be predicted to function as a physiologically important apoptosis suppressor when Bax, but not Bak, becomes activated. In this regard, gene ablation studies in mice in which *bax*, *bak* or both of the multidomain pro-apoptotic Bcl-2 family genes is disrupted indicate that either *bax* or *bak* is required for apoptosis induced by diverse stimuli in fibroblasts and embryonic stem cells [30,31]. However, analysis of the Bax and Bak proteins *in vivo* indicates that not all tissues simultaneously express these proteins [32–34]; thus circumstances presumably exist where Bcl-B is adequate to provide protection from apoptosis because Bak is not expressed. Evidence supporting this notion is found in studies of mice in which only the *bax* gene was ablated, showing, for example, a non-redundant role for the Bax protein in neuronal cell death induced by stroke and axotomy [35,36]. Evidence for an important role for the Bax protein has also been identified in human tumours that develop mutations in both copies of their *BAX* genes, correlating with loss of sensitivity to apoptosis induced, for example, by TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand) *in vitro* [37,38] and with poor patient prognosis [39]. Similarly, $bax^{-/-}$ mice display increased incidence of tumours under certain circumstances [40,41], indicating that nullifying *bax* alone can be sufficient to afford cells with a selective survival advantage and thus promote malignancy.

The Bcl-2 family comprises a large membership, with 24 and 27 genes identified in humans and mice respectively [12]. Tissue-specific roles for various members of this multigene family ($n = 24$ in humans) have been defined using gene knock-out experiments in mice [4], suggesting unique roles for individual Bcl-2 family proteins at discrete developmental stages. Based on nucleotide and amino-acid sequence comparisons, the closest homologue of Bcl-B in the mouse is Diva/Boo. Analysis of *boo/diva* knock-out mice showed no obvious histological abnormalities, and long-term survival did not differ from littermate controls [42]. Although *diva/boo* is highly expressed in ovary and testis, the $boo/diva^{-/-}$ mice are born at the expected ratios and both females and males are fertile [42]. However, it remains questionable whether *BCL-B* and *diva/boo* represent orthologous genes, given the striking differences in their patterns of expression *in vivo*, where mouse Diva/Boo is predominantly expressed in ovary and testis, being almost undetectable in other organs, whereas human Bcl-B is present in many organs, with the highest levels typically found in liver, pancreas, brain and lung [13,14]. Also, striking differences in the specificity of Bcl-B and Diva/Boo for BH3-containing dimerization partners has been reported, with human Bcl-B binding Bax, but not Bak, and mouse Diva/Boo binding Bak, but not Bax [13,17].

Pathological elevations in expression of Bcl-2 or Bcl-X_L are commonly observed in many leukaemias, lymphomas and solid tumours, including prostate, breast, lung and colorectal cancers [34,40,41,43]. We have also observed frequent expression of Bcl-B in human tumour cell lines (D. Zhai, unpublished work), although the relative importance of Bcl-B relative to other anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, remains to be determined. Recently, interest has emerged in the possibility of generating small-molecule drugs that target the BH3-binding pockets of anti-apoptotic Bcl-2 family proteins, mimicking the inhibitory effects of pro-apoptotic BH3 domains, and restoring apoptosis sensitivity to cancer cells that over-express proteins such as Bcl-2 and Bcl-X_L [12]. Indeed, several prototype chemical compounds have been described that compete for interactions with the BH3-binding pocket of either Bcl-2 or Bcl-X_L and that induce apoptosis of tumour cell lines in culture [44–46]. Through medicinal chemistry, compounds can be designed to either interact broadly with several anti-apoptotic Bcl-2 family members or selectively with only certain members of this family. The goal of such structure–activity relation studies is to determine which Bcl-2 family proteins are critical to inhibit for achieving efficacy, and which are desirable to avoid because of toxic side effects. Given the apparent differences in the BH3 domain specificity of Bcl-X_L and Bcl-B, as demonstrated in the present paper, it should be possible to design compounds that selectively target anti-apoptotic proteins, such as Bcl-2 or Bcl-X_L, without interfering with Bcl-B. For example, to avoid toxic side effects, it may be important to delineate whether compounds intended to antagonize Bcl-2 or Bcl-X_L also interact with Bcl-B, so that structure–activity relation studies can steer reactivity away from Bcl-B, while retaining activity against Bcl-2 or Bcl-X_L. Alternatively, if Bcl-B proves to be important for maintaining survival of tumour cells, then broad-spectrum compounds that target Bcl-B in addition to Bcl-2 and Bcl-X_L might be desired. We have observed, for example, by NMR methods that one of the previously described Bcl-X_L antagonists, BH3Is [45], also interacts with the BH3-binding pocket of Bcl-B (D. Zhai, unpublished work). Thus it would be interesting to contrast the anti-tumour activity and side-effect profiles of analogues of BH3Is that have been modified to either retain or lose Bcl-B-binding activity. The findings reported in the present study should help to contribute to those efforts.

We thank R. Cornell and J. Valois for manuscript assistance, and acknowledge the generous support of the National Institutes of Health (grants GM60554 and CA78040).

REFERENCES

1 Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. Science (Washington, D.C.) **267**, 1456–1462

2 Green, D. R. (1998) Apoptotic pathways: the roads to ruin. Cell (Cambridge, Mass.) **94**, 695–698

3 Reed, J. C. (2000) Mechanisms of apoptosis (Warner/Lambert Award). Am. J. Pathol. **157**, 1415–1430

4 Reed, J. C. (1997) Double identity for proteins of the Bcl-2 family. Nature (London) **387**, 773–776

5 Adams, J. and Cory, S. (1998) The Bcl-2 protein family: arbiters of cell survival. Science (Washington, D.C.) **281**, 1322–1326

6 Chao, D. T. and Korsmeyer, S. J. (1998) Bcl-2 family: regulators of cell death. Annu. Rev. Immunol. **16**, 395–419

7 Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkatachalam, M. A. and Saikumar, P. (2001) Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. J. Biol. Chem. **276**, 18361–18374

8 Wang, K., Gross, A., Waksman, G. and Korsmeyer, S. J. (1998) Mutagenesis of the BH3 domain of Bax identifies critical residues for dimerization and killing. Mol. Cell. Biol. **18**, 6083–6089

9 Kelekar, A. and Thompson, C. B. (1998) Bcl-2-family proteins – the role of the BH3 domain in apoptosis. Trends Cell Biol. **8**, 324–330

10 Zha, H. and Reed, J. C. (1997) Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. J. Biol. Chem. **272**, 31482–31488

11 Simonian, P. L., Grillot, D. A. and Nunez, G. (1997) Bak can accelerate chemotherapy-induced cell death independently of its heterodimerization with Bcl-XL and Bcl-2. Oncogene **15**, 1871–1875

12 Reed, J. C. (2003) Apoptosis-targeted therapies for cancer. Cancer Cell **3**, 17–22

© 2003 Biochemical Society

13 Ke, N., Godzik, A. and Reed, J. C. (2001) Bcl-B: A novel Bcl-2 family member that differentially binds and regulates Bax and Bak. J. Biol. Chem. **276**, 12481–12484
14 Aouacheria, A., Arnaud, E., Venet, S., Lalle, P., Gouy, M., Rigal, D. and Gillet, G. (2001) Nrh, a human homologue of Nr-13 associates with Bcl-Xs and is an inhibitor of apoptosis. Oncogene **20**, 5846–5855
15 Lee, R., Chen, J., Matthews, C. P., McDougall, J. K. and Neiman, P. E. (2001) Characterization of NR13-related human cell death regulator, Boo/Diva, in normal and cancer tissues. Biochim. Biophys. Acta **1520**, 187–194
16 Inohara, N., Gourley, T. S., Carrio, R., Muniz, M., Merino, J., Garcia, I., Koseki, T., Hu, Y., Chen, S. and Nunez, G. (1998) Diva, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death. J. Biol. Chem. **273**, 32479–32486
17 Song, Q., Kuang, Y., Dixit, V. M. and Vincenz, C. (1999) Boo, a novel negative regulator of cell death, interactst with Apaf-1. EMBO J. **18**, 167–178
18 Jürgensmeier, J. M., Krajewski, S., Armstrong, R., Wilson, G. M., Oltersdorf, T., Fritz, L. C., Reed, J. C. and Ottilie, S. (1997) Bax- and Bak-induced cell death in the fission yeast *Schizosaccharomyces pombe*. Mol. Biol. Cell **8**, 325–339
19 Zha, H., Aime-Sempe, C., Sato, T. and Reed, J. C. (1996) Pro-apoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. J. Biol. Chem. **271**, 7440–7444
20 Zhang, H., Nimmer, P., Rosenberg, S. H., Ng, S. C. and Joseph, M. (2002) Development of a high-throughput fluorescence polarization assay for Bcl-x_L. Anal. Biochem. **307**, 70–75
21 Dandliker, W. B., Hsu, M. L., Levin, J. and Rao, B. R. (1981) Equilibrium and kinetic inhibition assays based upon fluorescence polarization. Methods Enzymol. **74**, 3–28
22 Deveraux, Q. L., Takahashi, R., Salvesen, G. S. and Reed, J. C. (1997) X-linked IAP is a direct inhibitor of cell death proteases. Nature (London) **388**, 300–304
23 Jaroszewski, L., Rychlewski, L., Reed, J. C. and Godzik, A. (2000) ATP-activated oligomerization as a mechanism for apoptosis regulation: fold and mechanism prediction for CED-4. Protein Sci. **39**, 197–203
24 Sali, A. and Blundell, T. L. (1993) Comparative protein modeling by satisfaction of spatial restraints. J. Mol. Biol. **234**, 779–815
25 Vakser, I. A., Matar, O. G. and Lam, C. F. (1999) A systematic study of low-resolution recognition in protein–protein complexes. Proc. Natl. Acad. Sci. U.S.A. **96**, 8477–8482
26 Suzuki, M., Youle, R. J. and Tjandra, N. (2000) Structure of Bax: Co-regulation of dimer formation and intracellular localization. Cell (Cambridge, Mass.) **103**, 645–654
27 Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J. et al. (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science (Washington, D.C.) **275**, 983–986
28 Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Changs, B. S., Thompson, C. B., Wong, S. et al. (1996) X-ray and NMR structure of human Bcl-XL, an inhibitor of programmed cell death. Nature (London) **381**, 335–341
29 Petros, A. M., Medek, A., Nettesheim, D. G., Kim, D. H., Yoon, H. S., Swift, K., Matayoshi, E. D., Oltersdorf, T. and Fesik, S. W. (2001) Solution structure of the antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. U.S.A. **98**, 3012–3017
30 Lindsten, T., Ross, A. J., King, A., Zong, W., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K. et al. (2000) The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. Mol. Cell **6**, 1389–1399
31 Wei, M. C., Zong, W.-X., Cheng, E. H.-Y., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B. and Korsmeyer, S. J. (2001) Proapoptotic bax and bak: A requisite gateway to mitochondrial dysfunction and death. Science (Washington, D.C.) **292**, 727–730
32 Krajewski, S., Krajewska, M. and Reed, J. C. (1996) Immunohistochemical analysis of in vivo patterns of Bak expression, a pro-apoptotic member of the Bcl-2 protein family. Cancer Res. **56**, 2849–2855
33 Krajewski, S., Krajewska, M., Shabaik, A., Miyashita, T., Wang, H.-G. and Reed, J. C. (1994) Immunohistochemical determination of *in vivo* distribution of bax, a dominant inhibitor of Bcl-2. Am. J. Pathol. **145**, 1323–1333
34 Krajewski, S., Blomvqvist, C., Franssila, K., Krajewska, M., Wasenius, V.-M., Niskanen, E. and Reed, J. C. (1995) Reduced expression of pro-apoptotic gene Bax is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Cancer Res. **55**, 4471–4478
35 Knudson, C. M., Tung, K. S. K., Tourtellotte, W. G., Brown, G. A. J. and Korsmeyer, S. J. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science (Washington, D.C.) **270**, 96–99
36 Deckwerth, T. L., Elliott, J. L., Knudson, C. M., Johnson, J. E. M., Snider, W. D. and Korsmeyer, S. J. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. Neuron **17**, 401–411
37 Deng, Y., Lin, Y. and Wu, X. (2002) TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. Genes Dev. **16**, 33–45
38 LeBlanc, H., Lawrence, D., Varfolomeev, E., Totpal, K., Morlan, J., Schow, P., Fong, S., Schwall, R., Sinicropi, D. and Ashkenazi, A. (2002) Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the pro-apoptotic Bcl-2 homolog Bax. Nat. Med. **8**, 274–281
39 Knudson, C. M., Johnson, G. M., Lin, Y. and Korsmeyer, S. J. (2001) Bax accelerates tumorigenesis in p53-deficient mice. Cancer Res. **61**, 659–665
40 Reed, J. C. (1995) Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. Curr. Opin. Oncol. **7**, 541–546
41 Naik, P., Karrim, J. and Hanahan, D. (1996) The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors. Genes Dev. **10**, 2105–2116
42 Russell, H. R., Lee, Y., Miller, H. L., Zhao, J. and McKinnon, P. J. (2002) Murine ovarian development is not affected by inactivation of the Bcl-2 family member Diva. Mol. Cell. Biol. **22**, 6866–6870
43 Raffo, A. J., Perlman, H., Chen, M.-W., Day, M. L., Streitman, J. S. and Buttyan, R. (1995) Overexpression of bcl-2 protects prostate cancer cells from apoptosis *in vitro* and confers resistance to androgen depletion *in vivo*. Cancer Res. **55**, 4438–4445
44 Wang, J.-L., Liu, D., Zhang, Z.-J., Shan, S., Han, X., Srinivascula, A. M., Croce, C. M., Alnemri, E. S. and Huang, Z. (2000) Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc. Natl. Acad. Sci. U.S.A. **97**, 7124–7129
45 Degterev, A., Lugovskoy, A., Cardone, M., Mulley, B., Wagner, G., Mitchison, T. and Yuan, J. (2001) Identification of small-molecule inhibitors of interaction between the BH3 domain and Bcl-xL. Nat. Cell Biol. **3**, 173–182
46 Tzung, S., Kim, K. M., Basanez, G., Giedt, C. D., Simon, J., Zimmerberg, J., Zhang, K. Y. J. and Hockenbery, D. M. (2001) Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. Nat. Cell Biol. **3**, 183–192

Received 7 March 2003/21 July 2003; accepted 15 August 2003
Published as BJ Immediate Publication 15 August 2003, DOI 10.1042/BJ20030374

© 2003 Biochemical Society