Bcl-2 inhibits apoptosis induced by a wide variety of stimuli. In contrast, the Bcl-2 homologue, Bax, antagonizes Bcl-2's death protecting function. Bcl-2 forms protein-protein homodimers with itself and heterodimers with Bax, and previous experiments have shown that point mutations in Bcl-2 can abrogate Bax binding while leaving homodimerization intact. These mutagenesis results can be interpreted to suggest that Bcl-2 has separate binding sites that are responsible for homodimer and heterodimer formation. Results from yeast two-hybrid studies have also suggested that homodimerization and heterodimerization reflect distinct modes of interaction. However, using quantitative plate binding assays, we now show that Bax as well as peptides derived from the BH3 domains of Bax and Bak block both Bcl-2/Bax binding and Bcl-2/Bcl-2 binding. Similar assays demonstrate that Bcl-x\textsubscript{L} can form both homodimers and heterodimers and that these interactions are also inhibited by Bax and the BH3-derived peptides. These results demonstrate that the same binding motifs are responsible for both homodimerization and heterodimerization of Bcl-2 family members.

The regulation of programmed cell death is important for proper development and homeostasis in multicellular organisms (1, 2). Genetic studies have demonstrated that programmed cell death is controlled by a cell death pathway, and several key genes in this pathway have been identified (3, 4). One important set of genes, the Ice/ced-3 family, encodes cysteine proteases that are essential for effecting apoptotic death (5, 6). Another set of genes, the bcl-2 family, encodes proteins including Bcl-2 and Bcl-x\textsubscript{L}, that function to inhibit apoptosis (7–11), acting at a point in the death pathway that is upstream of the activation of at least some members of the ICE/CED-3 family (12, 13). The bcl-2 family also encodes the proteins Bax and Bak that promote cell death and can antagonize the cell protective functions of Bcl-2 (14–17). These Bcl-2 family members share significant sequence homology, particularly in regions known as BH1, BH2, and BH3 domains (18, 19).

Bcl-2 family proteins have been shown to form a variety of protein-protein interactions, both homodimers and heterodimers. For example, Bcl-2 can homodimerize with itself as well as form heterodimers with Bax (20). Similarly, Bcl-x\textsubscript{L} can form heterodimers with Bax and Bak; however, there has been controversy regarding the ability of Bcl-x\textsubscript{L} to homodimerize (15, 21–23). Mutagenesis studies have suggested that Bcl-2 family dimerization may be important for function. For example, the G145A mutation in the BH1 domain of Bcl-2 fails to heterodimerize with Bax and no longer can suppress apoptosis (20); similar results were observed with the homologous mutation in Bcl-x\textsubscript{L} (22). Interestingly, while several BH1 and BH2 mutants of Bcl-2 failed to heterodimerize with Bax, these mutations did not abrogate their ability to dimerize with wild-type Bcl-2 (20). This data, together with additional yeast two-hybrid data (19), has suggested that homodimer and heterodimer formation may reflect distinct binding processes utilizing distinct binding sites on the various molecules. In the present report, however, we use quantitative binding assays to demonstrate that homodimers and heterodimers are formed by interactions at the same sites.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Two expression vectors were used to express human Bcl-2, Bcl-x\textsubscript{L}, and Bax in bacteria. In all cases, the C-terminal membrane spanning domain in these proteins was deleted. pGEX-4T-1 (Pharmacia Biotech Inc.) was used for expression with N-terminal GST\textsuperscript{§} tags. The construct for expression of GST-Bcl-2 coding for amino acids 1–218 of human Bcl-2 was a generous gift from John Reed (Burnham Institute). For the expression of GST-Bcl-x\textsubscript{L}, a fragment coding for amino acids 1–211 was obtained from a full-length Bcl-x\textsubscript{L} cDNA template by PCR with Pfu polymerase (Stratagene). The primer sequences were: 5\textsuperscript{'} CTC GAG CTA CCA CGT GGG CGT CCC AAA 3\textsuperscript{'}, 5\textsuperscript{'} TCT GGC CAT ATG TCT CAG AGC AAC CGG 3\textsuperscript{'}, with EcoRI as a 5\textsuperscript{'} cloning site and XhoI as a 3\textsuperscript{'} cloning site.

Peptide Synthesis—Peptides were synthesized by standard solid phase methodology. Molecular weight was confirmed by mass spectrometry. Purity was considered acceptable when greater than 70% by high performance liquid chromatography system (Pharmacia) columns on a fast protein liquid chromatography system (Pharmacia) essentially following the manufacturer instructions.

The purity of proteins expressed by these methods was assessed by SDS-polyacrylamide gel electrophoresis (see Fig. 1). Protein concentrations were determined routinely using a bicinchoninic acid method (Pierce) with a bovine serum albumin (BSA) standard.

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1 The abbreviations used are: GST, glutathione S-transferase; 6H, 6-histidine; PBS, phosphate-buffered saline; PBS-T, PBS + Tween 20; BSA, bovine serum albumin.
pressure liquid chromatography analysis. Peptides not meeting this criterion were high pressure liquid chromatography purified. The following peptides were synthesized: human Bax 21-mer (52–72) ORAGRELRR (TO16); human Bax (G67R) 16-mer (72–87) biotinylated on the N terminus QGVRQALAIIDDRN (T01); human Bax (G67R) 21-mer (52–72) ORAGRELRR (T09); mouse Bad 16-mer (138–153) biotinylated on the N terminus PPNLWQAQRYGRELRR (T018).

In Vitro Protein-Protein Binding Assays—Purified 6H-Bax, 6H-Bcl-2 or 6H-Bcl-xL was diluted to 4 µg/ml in phosphate-buffered saline (PBS) and coated onto 96-well microtiter plates (50 µl/well; Immunosorb, Nunc) for 18 h at 4 °C. All other incubations were performed at room temperature. The plates were washed 2 times with PBS containing 0.05% (w/v) Tween-20 (PBS-T) and then blocked with 150 µl of 2% (w/v) BSA in PBS for 2 h. Subsequently, the plates were washed 2 times with PBS-T and incubated with a range of concentrations (20–0.0001 µM) of GST-Bcl-2 or GST- Bcl-xL in PBS-T containing 0.5% (w/v) BSA (50 µl/well), and the interaction was allowed to proceed for 2 h before washing the plates 5 times with PBS-T. The wells were incubated for 1 h with 50 µl of the primary antibody, a mouse anti-GST monoclonal antibody (7E5A6) (a kind gift of John Reed) used at 1 ng/ml in PBS-T plus BSA, before being washed 5 times with PBS-T. An alkaline phosphatase-conjugated goat anti-mouse antibody (Immunoresearch Laboratories) was added (50 µl/well) and incubated for 1 h, and the plates were then washed 5 times. p-Nitrophenyl phosphate (Kirkegaard and Perry) at 4 mg/ml in 10 mM diethanolamine (pH 9.5) containing 0.5 mM MgCl₂ was used as the enzyme substrate; the reaction was allowed to progress for 15 min and then stopped by the addition of 0.4 M NaOH (50 µl/well). The optical density at 405 nm was determined in a spectrophotometer (Molecular Devices).

Peptide and Protein Inhibition Assays—For the protein inhibition studies, the binding assay was carried out as above except for the following modifications. After the 96-well plates were coated with the appropriate proteins, the wells were then pre-incubated with increasing concentrations of 6H-Bcl-2, 6H-Bax, or 6H-Fadd (0.007–3.5 µM) before addition of the GST-tagged protein at a constant concentration (80 nm) throughout. This concentration was determined in preliminary experiments to be on the rate-limiting part of the binding curves. Similarly, peptide inhibition studies were carried out, as above, using increasing concentrations of the appropriate peptide (0.156–80 µM) for the pre-incubation.

RESULTS

Measurement of Heterodimer and Homodimer Formation—Quantitative solid-phase binding assays were created to measure the formation of Bcl-2/Bax and Bcl-xL/Bax heterodimers and Bcl-2/Bcl-2 and Bcl-xL/Bcl-xL homodimers. The requisite proteins were expressed in Escherichia coli as 6-histidine- or GST-fusion proteins and purified by affinity chromatography using Ni²⁺ chelation resin or glutathione-Sepharose, respectively. When analyzed by SDS-gel electrophoresis, these protein preparations yielded essentially single coomassie Blue-stained bands (Fig. 1). For each specific interaction, saturable binding was observed, whereas only low levels of background binding were seen when the control protein BSA was used as the solid phase binding partner (Fig. 2, A and B). To confirm that these in vitro interactions reflect the interactions observed in cells, we assessed the ability of the G145A and G138A point mutants of Bcl-2 and Bcl-xL, respectively, to bind to Bax; it has previously been shown by immunoprecipitation that these mutants fail to heterodimerize with Bax in cells. Recapitulating the cellular result, the mutants demonstrated a much reduced binding to Bax in the plate-binding assay (Fig. 2A). Further control experiments demonstrate that the observed binding properties are not dependent upon the nature of N-terminal tags (data not shown). Interestingly, while Bcl-xL forms homodimers in the plate-binding assay (Fig. 2B), Bcl-xL homodimers form weakly if at all by yeast two-hybrid analysis.

In vitro quantifying solid-phase binding partner (Fig. 2, binding were seen when the control protein BSA was used as stained bands (Fig. 1). For each specific interaction, saturable respectively. When analyzed by SDS-gel electrophoresis, these pro-

[21, 22] and have not been observed in immunoprecipitation assays in mammalian cells (23) or in structural studies with isolated protein (24).

The quantitative binding data allows an estimate of the binding affinity for the heterodimer and homodimer interactions. The concentration of liquid-phase protein necessary for half-maximal binding for each reaction is presented in Table I. Heterodimer and Homodimer Formation Is Inhibited by Either Bax or Bcl-2—If heterodimers and homodimers are formed by different sets of binding sites, then one would expect that Bax would only block heterodimer formation but would have no effect on formation of the homodimer. Alternatively, if both hetero- and homodimerization reflect binding at a common site on Bcl-2 or Bcl-xL, then Bax would be expected to block both interactions. To test this, we assessed the ability of 6H-Bcl-2, or 6H-Bax, to inhibit interactions in the plate binding assays. As expected, both Bcl-2 and Bax demonstrated dose-dependent inhibition of Bcl-2/Bax heterodimer formation (Fig. 3A) while control proteins (6H-mouse Fadd and casein) were inert. Similar results were obtained with the Bcl-xL/Bax heterodimer (Fig. 3B), demonstrating that Bcl-2 can cross-inhibit the related heterodimer interaction. Strikingly, Bax and Bcl-2 each inhibited the formation of Bcl-2 and Bcl-xL homodimers (Fig. 3, C and D). These results indicate that Bax contains the structural motif necessary for inhibition of homodimerization and further demonstrates the cross-competition between Bcl-2 and Bcl-xL. Quantitatively, the inhibition of heterodimer and homodimer formation by Bcl-2 and Bax showed similar concentration dependences (Table II).

BH3-derived Peptides Inhibit Both Heterodimer and Homodimer Formation—Mapping studies have indicated that Bax and Bak contain specific domains (BH3 domains) that are critical for their respective binding to Bcl-2 and Bcl-xL (18, 19). Based on the 7 helices observed within the crystal structure of Bcl-xL (24), these homologous BH3 regions are predicted to encompass the α2 helixes within Bax and Bak (24). To determine if disruption of BH3 binding is sufficient to disrupt the formation of the full protein-protein heterodimers, BH3-derived peptides were used as competitors in the plate-binding assays. A 16-mer peptide derived from the BH3 domain of Bak (amino acids 72–87), and encompassing its predicted α2 helix,

2 S. Ottilie, personal communication.
inhibited the formation of Bcl-2/Bax and Bcl-x\textsubscript{L}/Bax heterodimers, and similar inhibition was observed with a 21-mer peptide encompassing the homologous region in Bax (amino acids 52–72) (Fig. 4 A and B). In contrast, a control peptide derived from the sequence of mouse Bad and a BH3-derived peptide from Bax, but which contained a G67R substitution, failed to compete, demonstrating sequence specificity of the peptide inhibition. The Bax G67R substitution was observed as a naturally occurring mutation in the cell line HPB-ALL, derived from a patient with T-cell acute lymphoblastic leukemia (25).

Having demonstrated that BH3-derived peptides were capable of preventing heterodimerization, we next examined their effect on homodimerization. The Bak- and Bax-derived peptides were capable of inhibiting Bcl-2/Bcl-2 as well as Bcl-x\textsubscript{L}/Bcl-x\textsubscript{L} homodimer formation while control peptides were not (Fig. 4, C and D). These results demonstrate that homodimers, like heterodimers, form through BH3-dependent interactions. Quantitatively, the Bcl-2 interactions (Bcl-2/Bcl-2 and Bax/Bcl-2) showed similar IC\textsubscript{50} values for a given peptide inhibitor (Table II). The pair of Bcl-x\textsubscript{L} interactions (Bcl-x\textsubscript{L}/Bcl-x\textsubscript{L} and Bax/Bcl-x\textsubscript{L}) had IC\textsubscript{50} values that were similar to each other for a given peptide but that differed from the IC\textsubscript{50} values for the Bcl-2 interactions; the Bcl-x\textsubscript{L} interactions were more potently inhibited (Table II).

The conclusion that similar BH3-dependent interactions underly both homo- and heterodimer formation was further supported by analyzing the effects of a set of 8 nested Bax-derived BH3 peptides (Bax, amino acids 52–72, through Bax, amino acids 59–72) on Bcl-x\textsubscript{L}/Bcl-x\textsubscript{L} and Bax/Bcl-x\textsubscript{L} interactions. While the peptides with a length of 15 amino acids or greater retained significant inhibitory activity in both assays, activity abruptly fell off against heterodimer and homodimer when the length fell to 14 amino acids (Fig. 5). Thus, the length dependence of peptide inhibition is identical for both types of interaction, in accord with the existence of a common binding site responsible for both hetero- and homodimerization.

**DISCUSSION**

In the present study, we have investigated the binding properties of Bcl-2 family members with regard to homodimer and heterodimer formation. To perform these studies, we used recombinant Bcl-2, Bcl-x\textsubscript{L}, and Bax to construct reproducible solid-phase binding assays. We further validated the assays by
demonstrating that BH1 mutants of Bcl-2 and Bcl-xL lose their ability to bind to Bax in these assays as has previously been demonstrated in cells. Our data also provide the first estimate for the strength of these protein-protein interactions, although further efforts will be needed to obtain true $K_d$ values. Interestingly, Bcl-xL readily formed homodimers in our assay, whereas attempts by others to observe this interaction in cells or with isolated protein have failed. While we do not understand the basis for these differences, it is possible that a specific conformation of Bcl-xL is favored when bound to plastic and that this conformation facilitates homodimerization. For example, this conformation may alter the positioning of the a2 helix, facilitating its interaction with a second molecule of Bcl-xL.

The major objective of the present study was to determine if homodimers and heterodimers of Bcl-2 family members are formed by interactions at a common site or at different sites. Earlier data demonstrated that point mutants of Bcl-2 that lose their ability to heterodimerize with Bax can still form homodimers with wild-type Bcl-2, suggesting the possibility that distinct binding sites existed. Yeast two-hybrid analysis further suggested that homodimer and heterodimer formation utilized distinct binding domains, with the former occurring in an anti-parallel fashion and the latter in a parallel fashion (19, 26). However, our new data argues strongly that a common binding site is responsible for both interactions. First, Bax inhibited not only heterodimer formation but also formation of homodimers. If unique binding sites were responsible for the

![Graph A](image1)

**Fig. 3.** Bax and Bcl-2 are each capable of blocking both heterodimer and homodimer formation. A, wells were coated with 6H-Bax and incubated with a constant concentration (80 nM) of GST-Bcl-2 plus increasing concentration of either 6H-Bax (solid line, solid circles) or 6H-Bcl-2 (dashed line, solid squares) or the control protein 6H-Fadd (dotted lines, open triangles). B is the same as in panel A, except liquid phase contains a constant concentration (80 nM) of GST-Bcl-xL plus increasing concentrations of the competing proteins. C is the same as in panel A, except wells were coated with 6H-Bcl-2 instead of 6H-Bax. D is the same as in panel B, except wells were coated with 6H-Bcl-xL instead of 6H-Bax. Experiments were also performed using casein (Tropix) as a control inhibitor protein, and no inhibition was observed (data not shown). Bound protein was quantitated as in Fig. 2.

| Inhibitor | Bax/Bcl-2 | Bcl-2/Bcl-2 | Bax/Bcl-xL | Bcl-xL/Bcl-xL |
|-----------|-----------|------------|------------|--------------|
| 6H-Bcl-2  | 0.32 ± 0.06 | 0.99 ± 0.07 | 0.26 ± 0.02 | 0.46 ± 0.02 |
| 6H-Bax    | 0.49 ± 0.06 | 0.68 ± 0.25 | 0.71 ± 0.02 | 1.3 ± 0.3    |
| Bax (amino acids 52–72) | 18.7 ± 1.1 | 29.6 ± 0.6 | 5.2 ± 0.5 | 4.4 ± 0.6    |
| Bak (amino acids 72–87) | 9.4 ± 0.6 | 6.4 ± 1.7 | 0.08 ± 0.02 | 0.08 ± 0.00* |
| Bax (amino acids 52–72) (G67R) | >250 | >250 | >250 | >250 |
| Bad (amino acids 138–153) | >250 | >250 | >250 | >250 |

*$a$, value ± range for two determinations.
two types of interaction, then Bax would not be expected to inhibit the homodimer. Second, peptides derived from the BH3 domains of Bax and Bak inhibit both homodimer and heterodimer formation, demonstrating that this domain is responsible for both interactions. And third, the inhibitory peptides showed the same length dependence against both interactions,
further indicating that they inhibit heterodimers and homodimers by binding to a single binding site. The interactions involving Bcl-xL were more potently inhibited by each peptide than were the Bcl-2 containing interactions. However, for a given peptide, the IC_{50} for inhibiting Bcl-xL/Bcl-xL was very close to that for inhibition of Bax/Bcl-xL (Table II). Similarly, for a given peptide, the IC_{50} values for inhibiting Bcl-2/Bcl-2 and Bax/Bcl-2 were comparable (Table II). These quantitative relationships are in accord with the hypothesis that the peptides interact with a single site on Bcl-2 or Bcl-xL that prevents the subsequent binding of a BH3 domain provided by either Bax or another molecule of itself. Thus, our data support a model in which Bax, Bcl-2, or Bcl-xL provides a ligand consisting of its predicted α2 helix and which is bound by a single site in Bcl-2 or Bcl-xL. We cannot rigorously rule out the possibility that Bax contains a binding site for the BH3 domain of Bcl-2 and that this interaction contributes significantly to heterodimer formation. However, several observations render this possibility unlikely. First, the quantitative relationships described above do not support the idea of a peptide binding site on Bax. If the inhibitory peptides bound to a site on Bax, we would expect that a given peptide would have similar IC_{50} values for inhibition of the two Bax heterodimers, but this was not observed. Second, if Bax contained a binding site for the BH3 domains of Bcl-2 or Bcl-xL, we would expect that BH1 point mutations (G145A and G138A, respectively) would still bind avidly to Bax. However, it is possible that the residual weak interaction observed between Bax and Bcl-2 (G145A) (Fig. 2A) does in fact reflect such binding. Last, although it has been reported that mouse Bax can form homodimers (14, 19, 22), published studies indicate that human Bax fails to homodimerize in a yeast two-hybrid assay (27). This further supports the idea that the human Bax used in our studies has poor affinity as a receptor for BH3 domains of Bcl-2 family proteins. It is possible that Bax adopts a conformation distinct from the three-dimensional structure observed for Bcl-xL (24) and which favors the ability of it to act as a Bcl-xL donor. While our data indicate that Bcl-2 and Bcl-xL both have sites that bind BH3 domains, the quantitative inhibitory data further indicate that these binding sites are pharmacologically distinct. The BH3-derived peptides, especially the Bak-derived peptide TO16, were significantly more potent in blocking the Bcl-xL interactions compared with the Bcl-2 interactions. This observation suggests that the binding sites in Bcl-2 and Bcl-xL may differ in functionally important ways. These data are also in accord with yeast two-hybrid binding data that demonstrate strong binding of Bak to Bcl-xL but not to Bcl-2 (15).

Given our data demonstrating a common site on Bcl-2 that is responsible for both homodimerization and heterodimerization, there are two possible explanations for the observation that Bcl-2 (G145A) retains an ability to dimerize with wild-type Bcl-2 but fails to bind Bax (20). It is possible that the G145A mutation in the BH1 domain destroys the binding site but that the BH3 domain in this mutant simply binds to the intact binding site in the wild-type Bcl-2 molecule. However, it is also possible that the G145A mutation modifies the binding site, preventing the BH3 domain of Bax from binding but still allowing the binding of the BH3 domain of Bcl-2. Experiments are in progress to distinguish between these models.

The recently published three-dimensional structure of Bcl-xL demonstrates the presence of a hydrophobic cleft on the surface of the molecule, and it was suggested that this groove may represent the binding site for Bax and Bak (24). If so, our data further suggest that this groove also represents the binding site responsible for the observed Bcl-xL homodimerization.

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