Proapoptotic Protein Bax Heterodimerizes with Bcl-2 and Homodimerizes with Bax via a Novel Domain (BH3) Distinct from BH1 and BH2*

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Most members of the Bcl-2 protein family of apoptosis regulating proteins contain two evolutionarily conserved domains, termed BH1 and BH2. Both BH1 and BH2 in the Bcl-2 protein are required for its function as an inhibitor of cell death and for heterodimerization with the proapoptotic protein Bax. In this report, we mapped the region in Bax required for heterodimerization with Bcl-2 and homodimerization with Bax, using yeast two-hybrid and in vitro protein-protein interaction assays. Neither the BH1 nor the BH2 domain of Bax was required for binding to the wild-type Bcl-2 and Bax proteins. Moreover, Bax (ΔBH1) and Bax (ΔBH2) mutant proteins bound efficiently to themselves and to each other, further confirming the lack of requirement for BH1 and BH2 for Bax/Bax homodimerization. Bax/Bax homodimerization was not dependent on the inclusion of the NH2-terminal 58 amino acids of the Bax protein in each dimerization partner, unlike Bcl-2/Bcl-2 homodimers which involve head-to-tail interactions between the region of Bcl-2 where BH1 and BH2 resides, and an NH2-terminal domain in Bcl-2 that contains another domain BH4 which is conserved among antiapoptotic members of the Bcl-2 family. Similarly, heterodimerization with Bcl-2 occurred without the NH2-terminal domain of either Bax or Bcl-2, suggesting a tail-to-tail interaction. The essential region in Bax required for both homodimerization with Bax and heterodimerization with Bcl-2 was mapped to residues 59–101. This region in Bax contains a stretch of 15 amino acids that is highly homologous in several members of the Bcl-2 protein family, suggesting the existence of a novel functional domain which we have termed BH3. Deletion of this 15-amino acid region abolished the ability of Bax to dimerize with itself and to heterodimerize with Bcl-2. The findings suggest that the structural features of Bax and Bcl-2 that allow them to participate in homo- and heterodimerization phenomena are markedly different, despite their amino-acid sequence similarity.

Programmed cell death and apoptosis are active forms of cell suicide which play a variety of important roles under normal physiological conditions and which, when dysregulated, can contribute to several diseases including cancer, autoimmunity, AIDS, and ischemia-associated tissue loss (reviewed in Refs. 1 and 2). The Bcl-2 family proteins regulate a distal step in an evolutionarily conserved pathway for programmed cell death (1, 3). Several members of the Bcl-2 protein family can form physical interactions with each other in a complicated network of homo- and heterodimers (4–6). Although many details remain unclear at present, in general, the ratio between antiapoptotic proteins such as Bcl-2 relative to pro-cell death proteins such as Bax determines the ultimate sensitivity of cells to various apoptotic stimuli (7).

With the exception of some relatively nonabundant isoforms that arise through alternative mRNA splicing mechanisms, essentially all known members of the Bcl-2 protein contain two conserved regions of amino acid similarity, which we have previously termed Bcl-2 domains (BD) B and C but which are better known as BH1 and BH2 (4, 5, 8). In addition, the antiapoptotic proteins Bcl-2, Bcl-XL, Mcl-1, A1, Nrt-1, and Ced-9 all contain an additional region of homology near their NH2 termini, comprising a domain which we have termed BD-A but hereinafter refer to as BH4 for Bcl-2 homology-4 domain. Mutagenesis studies have shown that deletion of the BH1 or BH2 domain of Bcl-2 as well as certain amino acid substitutions in these conserved domains abolish Bcl-2 function as a suppressor of cell death and also abrogate the ability of Bcl-2 to form heterodimers with Bax (9, 10). Similar mutations in the BH1 and BH2 domains of the antiapoptotic protein Bcl-XL have the same effects on function and Bax binding (6). These observations suggest that for Bcl-2 and Bcl-XL to suppress apoptosis, they must be able to heterodimerize with Bax. However, the situation is likely to be more complicated because deletion of the BH4 domain of Bcl-2 also destroys function but does not interfere with Bax binding (10, 11).

In contrast to Bcl-2, essentially nothing is known at present about structure-function relations in the Bax protein as pertains to homodimerization with itself and heterodimerization with Bcl-2. In this report, we map for the first time a dimerization domain in the Bax protein and show that this novel domain, which we have termed BH3, is distinct from BH1 and BH2.

MATERIALS AND METHODS

A murine bax cDNA (12) was employed for mutagenesis experiments. Mutations were created using polymerase chain reaction-assisted methods and specific primers, essentially as described (5, 10, 13) (details available upon request), and their DNA sequences were confirmed by routine dideoxy sequencing methods. Bax mutants were expressed as fusion proteins either with an NH2-terminal LexA DNA binding domain in pEG202 for yeast two-hybrid experiments or with an NH2-terminal GST1 domain in pGEX-4T-1 for production of recombinant proteins. This work was generously supported by CaP-CURE, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: GST, glutathione S-transferase; X-Gal, 5-bromo-4-chloro-3-indoyl ß-D-galactoside.
BH3-dependent Dimerization of Bax

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RESULTS AND DISCUSSION

For initial explorations of the regions within the Bax protein required for homodimerization with Bax and heterodimerization with Bcl-2, a series of NH2-terminal and COOH-terminal truncation mutants were constructed in the two-hybrid plasmid pEG202 and expressed in yeast as fusion proteins with NH2-LexA DNA binding domains (Fig. 1). These LexA-Bax proteins were then tested by yeast two-hybrid assays for interactions with “full-length” Bax and Bcl-2 proteins (missing TM domains only), which were expressed as fusions with a NH2-transactivation domain (TA) under the control of a Gal-1 promoter using the plasmid pG4-5 (18).

The essentially full-length Bax protein (amino acids 1–171; i.e. missing only residues 172–COOH terminus to exclude TM domain) produced strong two-hybrid interactions with both Bax and Bcl-2 when plated on galactose-containing medium (induces Gal-1 promoter in pG4-5), whereas little or no growth on leucine or positivity in β-galactosidase colorimetric assays occurred when cells were plated on glucose-containing medium (represses Gal-1 promoter). COOH-terminal truncation mutants of Bax containing only residues 1–159 or 1–117 retained the ability to interact strongly with both Bax and Bcl-2, whereas a mutant that consisted only of amino acids 1–68 did not produce two-hybrid interactions with either full-length Bax or Bcl-2 (Fig. 1). Next, a series of NH2-terminal truncation mutants were tested. Deletion of the first 58 amino acids of Bax had no effect on its ability to form two-hybrid interactions with either Bax or Bcl-2. In contrast, removal of the first 99 or of the first 150 amino acids of Bax abolished all reactivity with both Bax and Bcl-2 (Fig. 1).

Because this result suggested that the dimerization domain of Bax lies between residues 59 and 117, a series of Bax mutants which contained only the 59–117 region or various subfragments thereof were tested for interactions with Bax and Bcl-2. The Bax-(59–117) fragment produced strong two-hybrid interactions with both Bax and Bcl-2, which were comparable in strength to those seen with full-length Bax. In contrast, a smaller fragment of Bax containing only amino acids 69–117 completely lacked reactivity with both Bax and Bcl-2 (Fig. 1). Since the 59–117 region of Bax retains the well-conserved BH1 domain, a smaller fragment of Bax was produced which lacked this domain, Bax-(59–101). This protein, however, resulted in high amounts of background Iac2 reporter gene transactivation, and thus could not be tested reliably by two-hybrid assay. A smaller fragment consisting of residues 69–101 was devoid of all reactivity with Bax or Bcl-2 in two-hybrid assays.

As an alternative to the Bax-(59–101) construct, an internal deletion mutant of Bax was produced which specifically lacked the sequences encoding the BH1 domain, Bax-(59–101). This protein, however, resulted in high amounts of background lacZ reporter gene transactivation, and thus could not be tested reliably by two-hybrid assay. A smaller fragment consisting of residues 69–101 was devoid of all reactivity with Bax or Bcl-2 in two-hybrid assays.

The mapping experiments described above suggested that the Bax dimerization domain resided between residues 59 and 101, but we were unable to test this through two-hybrid assays. For this reason, we explored the binding properties of Bax-(59-
101), as well as several other Bax mutants through in vitro binding assays where Bax mutants were expressed as GST fusions in bacteria and tested for specific binding to GST, \[^{35}S\]methionine-labeled Bax and Bcl-2. Alternatively, in some experiments, Bax mutants were in vitro translated and tested for binding to wild-type Bax or Bcl-2 GST-fusion proteins. As shown in Fig. 2A, a GST-Bax-(59–101) fusion protein bound to \[^{35}S\]Bax with comparable efficiency to an essentially full-length GST-Bax fusion protein (lacking only the TM domain). This 59–101 fragment of Bax bound effectively to in vitro translated Bcl-2 protein. The specificity of the binding was confirmed by use of control GST and GST-CD40 cytosolic domain fusion proteins, as well as by failure of the GST-Bax-(59–101) protein to interact with other irrelevant in vitro translated proteins, including R-Ras, Raf-1, and baculovirus p35 (Fig. 2A and data not shown).

Because the Bax-(59–101) fragment lacks the BH1 and BH2 domains, in vitro binding experiments were performed to confirm the lack of dependence in these conserved domains for dimerization with Bax and Bcl-2. In vitro translated full-length Bax was compared with Bax (ΔBH1) and Bax (ΔBH2) for binding in vitro to GST-Bax and GST-Bcl-2. Both the Bax (ΔBH1) and Bax (ΔBH2) mutants bound to GST-Bax and GST-Bcl-2 with efficiencies comparable to wild-type Bax, but did not bind to GST nonfusion, GST-CD40, or other control GST-fusion proteins (Fig. 2B and data not shown). In addition, the Bax (ΔBH2) and a Bax (Δ1–58) mutant were also capable of binding in vitro to a GST-Bax-(59–101) fusion protein, further indicating that the 59–101 region of Bax can bind to Bax independently of the BH2 and NH2-terminal domains of Bax (Fig. 2C). Interestingly, however, this 42-amino-acid fragment of Bax (residues 59–101) was not capable of binding to Bax (ΔBH1) in vitro. This result suggests that the BH1 domain, although perhaps not directly required for Bax homodimerization, may be necessary to facilitate the formation of an optimal binding site for the 59–101 Bax fragment (see below). In this regard, because the BH1 domain is located immediately adjacent to the 59–101 region, it is possible that BH1 is necessary for proper folding or contextual presentation of this region for homodimerization.

Finally, because the two-hybrid experiments above suggested an important role for residues 59–69 of Bax for dimerization with Bax and Bcl-2, a Bax-(59–117) fragment (which formed two-hybrid interactions with Bax and Bcl-2 in two-hybrid experiments) was compared with a Bax-(69–117) fragment (which did not react with Bax or Bcl-2 in yeast). When expressed as GST-fusion proteins and tested for binding in vitro to in vitro translated \[^{35}S\]-Bax and \[^{35}S\]-Bcl-2, the GST-Bax-(59–117) protein bound in vitro to Bax and Bcl-2 with efficiencies comparable to the essentially full-length GST-Bax protein which was missing only the TM domain (Fig. 2D). This result suggests that residues 59–69 of Bax are required for dimerization.
An amino acid sequence alignment was performed for the 59–69 region of Bax and several other known members of the Bcl-2 family, including Bcl-2, Bcl-X, Mcl-1, Ced-9, Bak, Bad, Bik, and Nr13. Significant homology was found in this region of Bax with the Bak, Bcl-2, Bcl-X, and Mcl-1 proteins (Fig. 3A), suggesting the existence of a functionally important, previously unrecognized conserved domain. We have termed this homologous region the Bcl-2 homology domain-3 (BH3). Thus, four evolutionarily conserved domains are envisioned in Bcl-2 family proteins: the BH4 domain (previously termed the A-box (5, 8, 10) which is found in the antiapoptotic members of the Bcl-2 family (Bcl-2, Bcl-XL, Mcl-1, Ced-9, A1, and Nr-13); the BH3 domain described here, and the BH1 and BH2 domains (Fig. 3B).

To confirm the importance of the BH3 domain for dimerization of Bax with itself and with Bcl-2, a Bax (ΔBH3) deletion mutant was tested for binding to Bax, Bax (ΔBH3), and Bcl-2 by two-hybrid assays. Although immunoblot analysis confirmed that the Bax (ΔBH3) protein was produced at levels comparable to the wild-type Bax protein (both when fused with a LexA-DNA binding domain or a B42 transactivation domain) (not shown), the Bax (ΔBH3) mutant failed to react with wild-type Bax, itself, or with Bcl-2 in two-hybrid assays (Fig. 4A). Thus, the BH3 domain is required for Bax homodimerization and for heterodimerization with Bcl-2.

An isoform of Bax (Bax-δ) has recently been described which arises because of alternative splicing (19). The predicted Bax-δ protein lacks residues 30–77 where the BH3 domain resides and thus should be incapable of dimerizing with either Bcl-2 or Bax, based on the findings shown here.

Our previous analysis of the domains required for Bcl-2/Bcl-2 homodimerization suggested an anti-parallel, head-to-tail model wherein the NH₂-terminal domain of Bcl-2 where the BH4 domain resides (residues 1–81) binds to structures contained in a downstream region of Bcl-2 (amino acids 83–218) (10). Furthermore, those studies showed that physical interactions of the NH₂-terminal Bcl-2 domain (residues 1–81) and downstream region in Bcl-2 (amino acids 83–218) require the simultaneous presence of both the BH1 and BH2 domain in the downstream region of Bcl-2 and the BH4 domain in the NH₂-terminal segment of Bcl-2 (10). However, if one dimerization partner was missing BH1 and BH2 (but retained BH4) and the other was lacking BH4 (but still had BH1 and BH2), then interactions could occur (10). This antiparallel fashion in which Bcl-2 homodimerizes promoted us to further explore the nature of Bax/Bax homodimers by testing binding of additional Bax mutants to each other by two-hybrid assays. As shown in Fig. 4A, a LexA DNA binding domain-Bax (ΔN) fusion protein containing Bax residues 59–171 formed strong interactions with a TA domain Bax-(59–171) fusion protein. Thus, the NH₂-termi-
nal 58 amino acids of Bax are not required for homodimerization of Bax (ΔN) to Bax (ΔN) mutants, implying that Bax/Bax homodimerization occurs via a tail-to-tail interaction. Fig. 4A shows in addition that the Bax (ΔBH1) and Bax (ΔBH2) internal deletion mutants were able to form strong two-hybrid interactions with themselves and each other, further confirming the data presented in Fig. 2C which suggested an absence of a requirement for BH1 or BH2 for homodimerization of Bax (Fig. 4A). The ability of the Bax (ΔBH1) mutant in particular to homodimerize with itself supports the idea that if BH1 is involved in Bax homodimerization, it plays only an indirect or facilitatory role compared to BH3. These same mutants of Bax (Bax (ΔN), Bax (ΔBH1), Bax (ΔBH2)) also formed two-hybrid interactions with Bcl-2 with strengths comparable to the wild-type Bax protein (not shown). The specificity of these interactions was confirmed by use of various irrelevant proteins (Fig. 4A; see legend).

Finally, the structural features of Bax/Bd-2 heterodimers were explored by testing the binding of Bd-2/mutants to Bax mutants. As shown in Fig. 4B, an in vitro translated N-truncation mutant of Bax missing the first 58 amino acids bound with comparable efficiencies in vitro to an essentially full-length GST-Bcl-2 protein (missing only TM domain; i.e. has residues 1–218) and a GST-Bd-2 N-truncation mutant missing the first 82 amino acids of the Bd-2 protein (B3–218) but failed to bind to a C-truncation mutant of Bd-2 comprised only of Bd-2 residues 1–81 fused to GST. In contrast, in vitro translated 35S-Bd-2 bound to both the GST-Bcl-2-(1–81) and GST-Bd-2-(B3–218) proteins employed for this experiment (not shown), confirming the integrity of the GST-Bcl-2-(1–81) protein despite its failure to bind to Bax. The specificity of the in vitro interactions of the Bax (ΔN) protein (residues 59→COOH terminus (191)) with GST-Bd-2 and GST-Bax was confirmed by use of GST, GST-CD40, and GST-R-Ras control proteins. Unlike Bd-2/Bd-2 homodimerization, therefore, the heterodimerization of Bd-2 with Bax appears to occur in a parallel, tail-to-tail fashion in which the NH2-terminal domains of neither Bd-2 nor Bax are required.

Taken together, these observations suggest that dimerization of Bax with itself and with Bd-2 occurs independently of the well-conserved BH1 and BH2 domains. Thus, the structural features of Bax that allow it to physically interact with other members of the Bd-2 family are strikingly different from Bcl-2, which does require BH1 and BH2 for homodimerization with Bax, as well as for homodimerization with itself, at least when testing for binding of mutant Bd-2 to mutant Bd-2 (9, 10). Moreover, the data presented here indicate that another region in the Bax protein, where the BH3 domain resides, is both necessary and sufficient for binding to the wild-type Bax and Bd-2 proteins and thus defines a novel dimerization domain for this family of apoptosis-regulating proteins. The finding that this region of Bax shares strong amino acid sequence homology with some other members of the Bd-2 protein family, including proapoptotic (Bak, Bik) and antiapoptotic (Bcl-2, Bcl-XL, Mcl-1) proteins, also raises the possibility that the BH3 domain may be required for interactions among other Bcl-2 family proteins. In this regard, while this paper was under review, a report appeared showing that the region of Bak that contains BH3 is sufficient both for heterodimerizing with Bcl-XL and for promoting apoptosis in mammalian cells (20). It remains to be determined however whether the BH3 domain of Bak promotes apoptosis by directly engaging the cell death pathway (i.e. effector domain) versus by acting as a decoy (i.e. regulatory domain) that ties up antiapoptotic protein such as Bcl-XL thereby preventing them from forming effective interactions with the full-length endogenous Bak or Bax proteins. In addition, a new member of the Bd-2 protein family was described Bik that: (a) promotes apoptosis, (b) contains a region with strong homology to the BH3 domain but lacks the BH1 and BH2 domains (Fig. 3, A and B), and (c) which binds to Bd-2 in a BH3-dependent manner. When taken together with the data presented here, therefore, these findings confirm the functional importance of the BH3 domain. It will be interesting in future investigations to determine whether the BH3-mediated homodimerization of Bax with itself is necessary for promotion of apoptosis by the Bax protein.

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