BH3 Domain of BAD Is Required for Heterodimerization with BCL-X\textsubscript{L} and Pro-apoptotic Activity*

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BAD interacts with anti-apoptotic molecules BCL-2 and BCL-X\textsubscript{L} and promotes apoptosis. BAD is phosphorylated on serine residues in response to a survival factor, interleukin-3. Phosphorylated BAD cannot bind to BCL-X\textsubscript{L} or BCL-2 at membrane sites and is found in the cytosol bound to 14-3-3. The BH3 domain of BAD required for heterodimerization with BCL-X\textsubscript{L} and its death agonist activity. Substitution of the conserved Leu\textsuperscript{151} with Ala in this predicted amphipathic \(\alpha\)-helical BH3 domain abrogated both functions.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Site-directed Mutagenesis—Standard polymerase chain reactions were used to amplify DNA. The Bad DNA fragments (141–204, 152–204, 163–204, 173–204, 141–183, and 141–194) were engineered with HindIII and EcoRI sites were cloned into pcDNA3 (Invitrogen) in frame with T7 and cytomegalovirus promoters. The site-directed mutagenesis of bad and bcl-2 (BAGD148A, BADR149A, BADL151A, and BCL-2L97A) was performed in pGEM-3Z derivatives using a QuikChange site-directed mutagenesis kit (Stratagene). The sequence confirmed mutant DNA was subcloned into the pSSPV expression vector.

Binding \(^{32}\text{P}\)-labeled BCL-2 to BAD Deletion Mutants—Total cell lysates were prepared from BL21 cells (Novagen), which contained a pET17b vector with BAD deletion constructs fused in frame with T7 gene 10 following induction with isopropyl-\(\beta\)-D-galactopyranoside (0.1 mM) for 1 h. Lysates (40 \(\mu\)g) were size fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane has hybridized with \(^{32}\text{P}\)-labeled GST-BCL-2 according to the protocol of Blanar and Rutter (13).

In Vivo Transcription-Translation and in Vitro Binding Assay—WT and mutant BAD and BCL-2 proteins were generated by an in vitro transcription-translation system (Promega). In vitro binding assays were performed as described previously (12).

Transfection, Viability Assay, and Cellular Fractionation—Stable transfectedants were generated in FL5.12 BCL-X\textsubscript{L} cells as described previously (12). Transient transfections were performed in BCL-deficient murine embryonic fibroblasts with pcDNA3-derived constructs. The luciferase reporter plasmid (0.1 mg) was mixed with 0.05 mg of various constructs and 3 \(\mu\)l of lipofectAMINE (Life Technologies, Inc.) in a volume of 0.5 ml added to murine embryonic fibroblast cells for 5 h. Cells were lysed 18–20 h later, and luciferase assays were performed using a standard substrate (Promega). Luciferase activities were quantified by a luminometer (OptocompII, MGM Instruments Inc.). An assessment of cell viability was displayed as the relative luciferase activity of a test construct compared with the control pcDNA3 plasmid (10). The viability of FL5.12 cells was measured by propidium iodide exclusion. FL5.12 BCL-X\textsubscript{L}/BAD cells were fractionated to separate cytosol from crude membrane as described previously (12).

Immunoprecipitation and Western Blots—Immunoprecipitation and Western blots were performed as described previously (12). The BAD

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1 The abbreviations used are: IL, interleukin; WT, wild type; Ab, antibody; mAb, monoclonal antibody; GST, glutathione S-transferase.
Abs used in this study include a rabbit polyclonal Ab (#10929) (11) and a hamster monoclonal Ab (2G11) (12). BCL-X L Abs include a rabbit polyclonal Ab (13.6) and a murine mAb (7B2) against human BCL-X L (14). The anti-14-3-3 Ab was from Upstate Biotechnology Inc.

RESULTS

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FIG. 1. The BH1/BH3 region of BAD is required for heterodimerization with BCL-2. A, map of a nested set of BAD deletion mutants indicating retained amino acids and position of BH1/BH3 and BH2 domains. B, binding of 32P-labeled GST-BCL-2 to BAD mutants on nitrocellulose in the autoradiogram of upper panel. The lower panel is a Coomassie Blue-stained gel showing approximately equal loading of BAD mutant proteins.

FIG. 2. Primary sequence homology and predicted structure of BAD BH3 domain. A, sequence homology within BH3 domains of human BAK (hBAK), human BIK (hBIK), murine BID (mBID), murine BAX (mBAX), and murine BAD (mBAD). The identical amino acids shared with BAD are boxed. The YGR minimal homology to BH1 found in BAD is noted. B, views of the hydrophobic surface (left) and polar surface (right) of the BH3 amphipathic α-helix of BAD (151–163) were calculated and displayed using GRASP (18). The surface is colored deep blue (23k_BT) in the most positively charged regions and deep red (−21k_BT) in the most negative, with linear interpolation for values in between. This model was generated using the protein building module (BUILDER) of INSIGHT II (Biosym, San Diego) and minimized using DISCOVER, the force field simulation module.

FIG. 3. The BH1/BH3 region is essential for pro-apoptotic function. A, BAD deletion mutants indicating retained amino acids and position of BH1/BH3 and BH2 domains. B, expression constructs of Wt BAD and each BAD mutant (pcDNA3 derivatives) were transiently co-transfected with a luciferase reporter vector into bad-deficient murine embryonic fibroblasts as described previously (10). The relative luciferase activity (%) is the ratio of a test construct over that of an empty pcDNA3 vector (100%). The means ± SD of three experiments are shown. The binding capacity of each truncated BAD molecule with either BCL-2 or BCL-X L was tested in an in vitro binding assay described in Fig. 5.

Abs used in this study include a rabbit polyclonal Ab (#10929) (11) and a hamster monoclonal Ab (2G11) (12). BCL-X L Abs include a rabbit polyclonal Ab (13.6) and a murine mAb (7B2) against human BCL-X L (14). The anti-14-3-3 Ab was from Upstate Biotechnology Inc.

RESULTS

BH3 domain of BAD is required for cell death, and heterodimerization-BCL-2 interaction cloning had identified three independent clones of Bad, the smallest encoding only residues 141–204, the C terminus (11). To define the minimal region in BAD essential for its interaction with BCL-2, we generated a nested set of deletion mutants (Fig. 1A). These mutant BAD proteins were expressed in vitro and tested for their ability to interact with BCL-2 protein. Removal of an additional 12 amino acids (BAD construct 152–204) abrogated binding to BCL-2 (Fig. 1B), whereas deletion of 32 amino acids from the C terminus (BAD 141–172) still bound BCL-2. Therefore, a small 31-amino acid region (141–172) is both sufficient and essential for BAD to heterodimerize with BCL-2. This includes a sequence (151–159) with homology to BH3 domains found in other pro-apoptotic molecules (Fig. 2A). The BH3 domain of BAD is predicted to be an amphipathic α-helix (Fig. 2B).

A transient transfection assay was used to assess the role of various regions of BAD in promoting apoptosis. A large N-terminal deletion mutant (127–204) with an intact BH1/BH3 region and a small C-terminal deletion mutant that removed the BH2 domain (1–181) were nearly as effective as Wt BAD in promoting cell death (Fig. 3). In contrast, deletion of the BH1/
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BH3 region (deletion of amino acids 142–165) diminished death-promoting function substantially. This same construct (BAD Δ142–165) also failed to bind BCL-2 or BCL-X<sub>L</sub> (Fig. 3B and data not shown). Thus, the BH1/BH3 region (142–165) is required for both heterodimerization and death agonist activity.

To further dissect this BH1/BH3 region we used site-directed mutagenesis to substitute individual amino acids (143–153) and assess the effect of their substitution. Of note, the greatest impact was not from substitutions in the region homologous to BH1 as had been noted for BCL-2 and BCL-X<sub>L</sub> (4, 3, 15, 16). Instead, replacement of Leu<sup>151</sup> of the BH3 domain with alanine (L151A) reduced the binding of mutant BAD with either BCL-2 or BCL-X<sub>L</sub> by more than 90%. In contrast, BAD G148A binding with BCL-2 was reduced approximately 50%, whereas its interaction with BCL-X<sub>L</sub> was only minimally affected (Fig. 4A).

We also assessed the domains in BCL-2 most involved in heterodimerization with BAD. An <i>in vitro</i> binding assay revealed that GST-BAD still interacts with slightly reduced efficiency to the BH1 mutant ml-3 (G145A) and weakly to the BH2 mutant ml-1 (W188A) but not at all to the BCL-2 BH3 mutant (L97A) (Fig. 5). Thus, BH3 plays a prominent role in heterodimerization for both the death agonist and antagonist.

To assess the death-promoting action of the BAD point mutants, we stably expressed them in the hematopoietic cell line FL5.12 BCL-X<sub>L</sub>. Clones with similar levels of Wt and mutant BAD as well as BCL-X<sub>L</sub> were identified (Fig. 4B) and tested for viability after IL-3 withdrawal. Mutants of BAD G148A and BAD R149A within the BH1-like region like Wt BAD reversed the protective effect of BCL-X<sub>L</sub>; however, a BH3 mutant BAD L151A could no longer promote cell death (Fig. 6C). The 7B2 anti-BCL-X<sub>L</sub> mAb co-precipitated the Wt BAD, BAD G148A, and somewhat less efficiently BAD R149A, but failed to co-precipitate substantial amounts of BAD L151A with BCL-X<sub>L</sub> (Fig. 4C). Consistent with this, a markedly increased amount of BAD L151A was present in the supernatant of this immunoprecipitate. This provides <i>in vivo</i> confirmation (Fig. 4C) for the loss of BAD L151A binding to BCL-X<sub>L</sub> noted <i>in vitro</i> (Fig. 4A).
Because intracellular localization of BAD is associated with its functional activity (12), we tested whether BAD L151A had also altered its subcellular distribution. The majority of BAD L151A was present in the cytosolic fraction with a more prominent upper band (Fig. 6A, lane Cyt, L151A), which represents the hyperphosphorylated form of BAD. Furthermore, anti-BAD mAb 2G11 co-precipitated significantly more 14-3-3 protein associated with BAD L151A than with Wt BAD or the other mutants (Fig. 6B). These data indicate that BAD L151A that is incapable of binding to BCL-X<sub>L</sub> is also functionally inactive and localized to the cytosol where it is bound to 14-3-3.

**DISCUSSION**

A combination of deletion mapping and site-specific mutagenesis identified a BH3 domain in BAD as critical for both its heterodimerization with BCL-2 or BCL-X<sub>L</sub> and its death-promoting activity. The short BH3 motif LR<sub>R</sub>MSDEFE proved most similar to BAX (Fig. 2A). Molecular modeling of the BH3 domain of BAD revealed a classic amphipathic α-helix in which the critical Leu<sup>151</sup> resides on the hydrophobic face (Fig. 2B). The close proximity of the BH3 motif of BAD to the previously noted BH1-like YGR core residues is somewhat unusual (Fig. 2A), and these YGR residues may reside within the same α-helix. Of note, BAK contains VGR residues in the N terminus of its amphipathic α2-helix proximal to the highly conserved BH3 motif (Fig. 2A). A mutant BAK R76A peptide demonstrated decreased binding to BCL-X<sub>L</sub> (17). This also suggests that the BAD structure will vary from the BCL-X<sub>L</sub> hydrophobic pocket generated by BH1, BH2, and BH3 (15). Of note substitution of Gly<sup>149</sup> of BAD affected its binding to BCL-2 much more than to BCL-X<sub>L</sub> indicating a difference in the contact sites of these anti-apoptotic molecules.

These data for BAD coincide with a growing body of evidence that implicates BH3 domains in pro-apoptotic molecules. The BH3 region of BAX and BAK are necessary for binding to BCL-X<sub>L</sub> and promoting apoptosis (5–7, 16). A detailed NMR analysis of wild type and mutant peptides of the BH3 amphipathic α2-helix of BAK indicated critical interactions with BCL-X<sub>L</sub> through both hydrophobic and electrostatic interactions (17). Moreover, the death agonists BIK and BID possess only the BH3 region, arguing that it represents the minimal death domain (8–10).

One model of BAD regulation holds that phosphorylated BAD would be inactive sequestered in the cytosol by 14-3-3, whereas nonphosphorylated BAD would be the active form bound to BCL-X<sub>L</sub>, inhibiting it and/or releasing pro-apoptotic BAX (12). However, this prior study of BAD phosphorylation did not address another model in which unbound, free BAD might serve as an active death agonist. The current data disfavor this model in that the minimal L151A point mutation of BH3 indicates that a BAD molecule that is unable to bind BCL-X<sub>L</sub> or BCL-2 is heavily phosphorylated, bound to 14-3-3 in the cytosol, and functionally inactive.

The prominence of the BH3 domain for BAD function combined with only minimal conservation of BH1 and BH2 motifs argue that BAD is more closely related to BID and BIK. Like BID, BAD lacks the C-terminal signal anchor sequence and may represent a death ligand that inhibits the membrane receptors BCL-2 and BCL-X<sub>L</sub>.

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