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Bad, a Heterodimeric Partner for Bcl-x_L and Bcl-2, Displaces Bax and Promotes Cell Death

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Summary

To extend the mammalian cell death pathway, we screened for further Bcl-2 interacting proteins. Both yeast two-hybrid screening and λ expression cloning identified a novel interacting protein, Bad, whose homology to Bcl-2 is limited to the BH1 and BH2 domains. Bad selectively dimerized with Bcl-x_L as well as Bcl-2, but not with Bax, Bcl-x_S, Mcl-1, A1, or itself. Bad binds more strongly to Bcl-x_L than Bcl-2 in mammalian cells, and it reversed the death repressor activity of Bcl-x_L, but not that of Bcl-2. When Bad dimerized with Bcl-x_L, Bax was displaced and apoptosis was restored. When approximately half of Bax was heterodimerized, death was inhibited. The susceptibility of a cell to a death signal is determined by these competing dimerizations in which levels of Bad influence the effectiveness of Bcl-2 versus Bcl-x_L in repressing death.

Introduction

The development of multilineage organisms and the maintenance of homeostasis within tissues both require a tightly regulated cell death pathway. However, the capacity of an individual cell to execute a suicidal response following a death stimulus can vary markedly during its differentiation. Both positive and negative regulators of apoptosis have been identified. The *bcl-2* proto-oncogene counters a variety of apoptotic stimuli (Vaux et al., 1988; Strasser et al., 1991; Garcia et al., 1992), indicating that it is a central death repressor acting well downstream in this pathway. In its gain of function form, CED-9, the homolog of Bcl-2 in Caenorhabditis elegans, represses the death of all 131 cells normally destined to die during the development of the nematode. Transgenic worms expressing Bcl-2 indicate that Bcl-2 can substitute for CED-9 in opposing some of these deaths (Vaux et al., 1992; Hengartner and Horvitz, 1994). Thus, evidence exists for an evolutionarily conserved cell death pathway that, in its basic tenets, may be common to all multicellular organisms.

An expanding family of Bcl-2-related proteins has recently been noted to share homology that is principally, but not exclusively, clustered within two conserved regions entitled Bcl-2 homology domains 1 and 2 (BH1 and BH2) (Williams and Smith, 1993; Yin et al., 1994). This includes Bax, Bcl-x_L, Mcl-1, A1 (Oltvai et al., 1993; Boise et al., 1993; Kozopas et al., 1993; Lin et al., 1993), and several open reading frames in DNA viruses. Several of these have been shown to regulate cell death, including Bcl-x_L, which represses apoptosis, and its short form Bcl-x_S, which favors cell death. Bax has been shown to homodimerize as well as heterodimerize with Bcl-2. When in excess, Bax counters the ability of Bcl-2 to repress cell death (Oltvai et al., 1993). Mutagenesis analysis of the BH1 and BH2 domains in Bcl-2 noted single amino acid substitutions that disrupted Bcl-2–Bax heterodimers, but not Bcl-2–Bcl-2 homodimers. The Bcl-2 mutants that failed to complex with Bax could no longer inhibit apoptosis (Yin et al., 1994). These data argue that this family functions in part through protein–protein interactions.

Analysis of the known Bcl-2 family members in a yeast two-hybrid assay demonstrated selective dimerizations. Wild-type Bcl-2, but not the BH1 mutants, interacted with Bax, validating the yeast assay as being reflective of functionally significant events in mammalian cells (Sedlak and S. J. K., unpublished data). We reasoned that additional Bcl-2-interacting proteins might exist that would further elucidate the cell death pathway. We utilized Bcl-2 protein to screen cDNA libraries in yeast two-hybrid and λ expression cloning. Both approaches identified the same distant *bcl-x_L*/bcl-2-associated death promoter homolog, *bad*, which was conserved within the BH1 and BH2 domains. Bad selectively heterodimerizes with Bcl-x_L as well as Bcl-2, but not with other family members. When Bad heterodimerizes with Bcl-x_L in mammalian cells, it displaces Bax from Bcl-x_L and promotes cell death. This argues that the set point of susceptibility to death in such a cell is determined by the relative levels and interactions among this trilogy of proteins.

Results

Isolation of Bad

The yeast two-hybrid system and λ expression cloning were used to screen for Bcl-2-interacting proteins. The bait plasmid was constructed by fusing Bcl-2, deleted of the C-terminal signal–anchor sequence to ensure translocation to the nucleus, to the GAL4 DNA-binding domain. For a positive control, Bax was fused to the GAL4 activation domain. The Bcl-2 bait plasmid was used to screen an oligo(dT)-primed mouse embryonic day 14.5 cDNA fusion library in a GAL4-activating domain vector (Chevray and Nathans, 1992). Transformants (400,000) were screened by the X-Gal filter assay. Sequence analysis of cDNA plasmids rescued from 12 positive yeast colonies revealed that four plasmids represented two independent clones of the same gene.

For λ expression cloning, a GST–Bcl-2 fusion protein containing a 5 amino acid heart muscle kinase domain (Blanar and Rutter, 1992) was in vitro labeled with [γ-^{32}P]ATP and was used to screen an oligo(dT)-primed mouse embryonic day 16 Ex-lox library (Novagen). Of 750,000 plaques, 12 positives represented three overlapping clones of the same gene isolated from yeast two-hybrid screening.

bad cDNA and the Protein Sequence It Encodes

The full-length cDNA sequence was determined from newborn mouse brain and adult mouse thymus libraries. The gene encodes a previously undescribed protein of 204 amino acids with a predicted molecular weight of 22.1 kDa. We will refer to this gene as *bad*. Comparison of the Bad protein sequence with known Bcl-2 family members revealed limited homology in the BH1 and BH2 domains (Figure 1). Specifically, Bad contains the highly conserved and functionally significant W/YGR triplet in BH1, the W at position 183, and the WD/E at the conserved exon juncture in BH2 (Yin et al., 1994). The spacing between the conserved BH1 and BH2 domains in Bad is also similar to that of the other Bcl-2 homologs. The shortest Bad clone that interacted with Bcl-2 possessed the BH1 and BH2 regions, suggesting that these conserved amino acids may be important for binding to Bcl-2 (Figure 1). Unlike the other known Bcl-2 family members, Bad does not have a predicted C-terminal signal–anchor sequence, suggesting that it may not exist as an integral membrane protein. Like the Bcl-2 homolog Mcl-1, Bad contains two predicted PEST sequences flanked by arginine or arginine and histidine residues (Figure 1) (Rogers et al., 1986). Of note, Bad contains a large number of charged residues (25 arginines plus lysines, 23 aspartates plus glutamates), with an estimated isoelectric point of 9.7 and an estimated charge of 2.47 at neutral pH. PROSITE analysis identified several potential phosphorylation and myristoylation sites. BLAST searches revealed no further homologies in the existing data bases.

Interaction of Bad with Other Bcl-2 Family Members

The full-length *bad* cDNA was cloned into yeast two-hybrid vectors and was tested for interaction with the mammalian Bcl-2 homologs by both X-Gal filter and liquid ONPG assays. Bad interacted strongly with Bcl-x_L and Bcl-2 in both the DNA-binding domain and activation domain plasmids. Bad did not interact with Bax, Bcl-x_S, Mcl-1, or A1. Unlike Bcl-2 or Bax, analysis in yeast two-hybrid suggests that Bad does not homodimerize (data not shown). Of note, the 1.1 kb *bad* RNA species is coexpressed with *bcl-x$_L$* in many tissues (data not shown).

Bad Heterodimerizes with Bcl-2 and Bcl-x_L in Mammalian Cells

An expression plasmid was constructed placing *bad*, with a 9 amino acid hemagglutinin (HA) epitope tag at the N-terminus, under the control of the splenic focus-forming virus (SFFV) long terminal repeat (Fulbrigge et al., 1988). The resultant pSFFVHA–Bad plasmid was stably transfected into the interleukin-3-dependent (IL-3-dependent) cell line FL5.12 and into FL5.12 clones already overexpressing Bcl-2 (Nuñez et al., 1990) or Bcl-x_L (Boise et al., 1993), generating FL5.12HA–Bad clones, FL5.12Bcl-2HA–Bad clones, and FL5.12Bcl-x_LHA–Bad clones. The expression levels of HA–Bad in these clones was determined by Western blot analysis using either the monoclonal antibody (MAb) to the HA tag (12CA5) or a novel polyclonal antibody to Bad (anti-Bad Ab). While the predicted molecular weight of HA–Bad was 23.4 kDa, it migrated at 30 kDa in SDS–polyacylamide gel electrophoresis (SDS–PAGE). This apparent molecular weight was also noted with bacterially produced protein, indicating that the anomalous migration is more likely due to primary structure rather than posttranslational modification. The FL5.12Bcl-2HA–Bad#4 and FL5.12Bcl-x_LHA–Bad#7 clones, which expressed comparable amounts of Bad (Figure 2A), were analyzed for in vivo heterodimerizations.

Immunoprecipitation of Bad, Bcl-2, and Bcl-x_L were per-

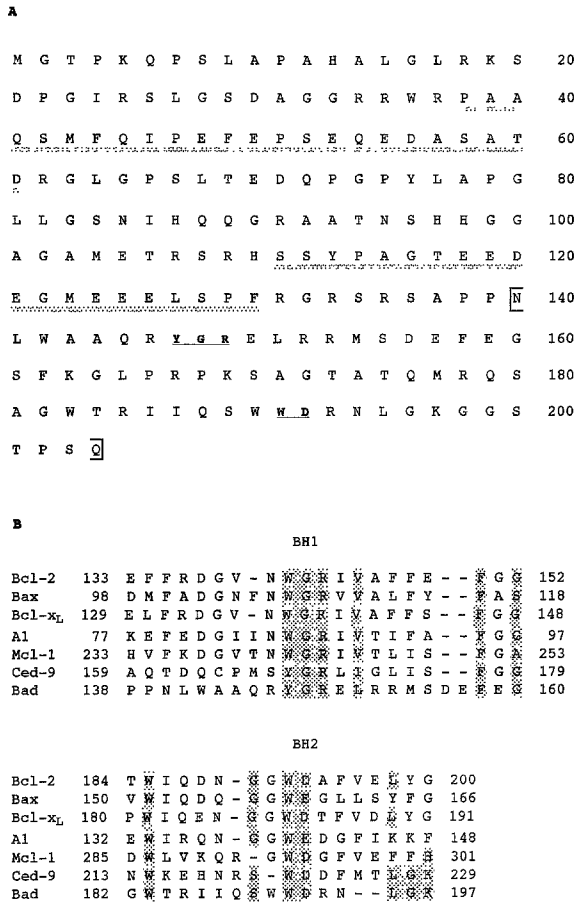

Figure 1. Sequence of Murine *bad*

(A) The predicted amino acid sequence of *bad*. Bold and underlined residues are conserved amino acids in BH1 and BH2. Dotted underlined amino acids indicate PEST sequences. Brackets indicate the shortest clone identified by Bcl-2 interactive screening.

(B) Alignment of Bad and Bcl-2 homologs in BH1 and BH2 domains. Numbers denote amino acid positions. The most conserved amino acids in BH1 and BH2 are stippled. Dashes denote gaps in the sequence to maximize alignment.

Abbreviations: kD, kilodaltons.

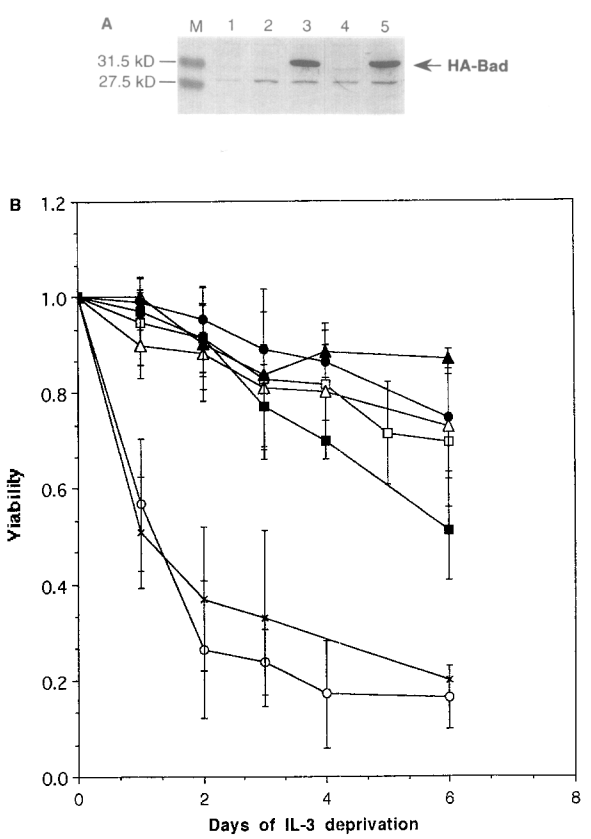

Figure 2. Western Blot Analysis and Viability of Bad-Expressing FL5.12 Clones

(A) Protein lysate (75 μg) was loaded in each lane. The anti-HA MAb 12CA5 was used to detect HA–Bad. The blot was developed with diaminobenzidine. Lane 1, FL5.12Neo; lane 2, FL5.12Bcl-x_L; lane 3, FL5.12Bcl-x_LHA–Bad#7; lane 4, FL5.12Bcl-2; and lane 5, FL5.12Bcl-2HA–Bad#4.

(B) Viability assays. Independent clones indicated by the symbols were deprived of IL-3, and the fraction of viable cells (with horizontal bars indicating ± SEM) was assessed by trypan blue exclusion. Data points were obtained from three or more experiments.

Symbols: open circle, FL5.12Neo; open square, FL5.12Bcl-2; closed circle, FL5.12Bcl-2HA–Bad#4; open triangle, FL5.12Bcl-2HA–Bad#15; closed triangle, FL5.12Bcl-x_LHygro; cross marks, FL5.12Bcl-x_LHA–Bad#7; closed square, FL5.12Bcl-x_LHA–Bad#8.

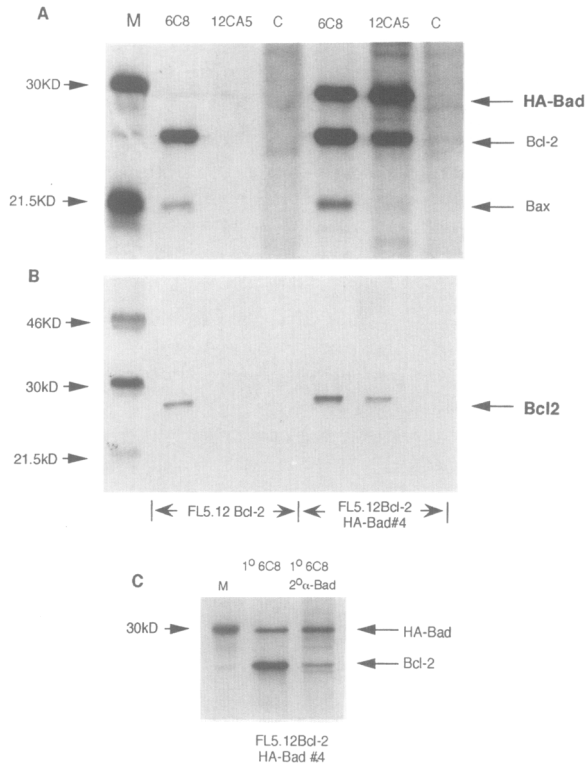

Figure 3. Coimmunoprecipitation of Bcl-2 and Bad

(A) Primary immunoprecipitation of the ^{35}S-labeled FL5.12Bcl-2 line (lanes 2, 3, 4) and FL5.12Bcl-2 transfected with pSFFVHA–Bad#4 (lanes 5, 6, 7). 6C8 is a MAb specific for human Bcl-2, and 12CA5 is a MAb against the HA epitope. One half of each sample was loaded on a 12.5% SDS–PAGE gel and fluorographed.

Abbreviations: C, isotype control Ab; KD, kilodaltons; M, marker lane.

(B) One half of each sample from (A) was used in Western blotting with 6C8 MAb and was developed with enhanced chemiluminescence.

Abbreviations: KD or kD, kilodaltons.

(C) Shown here is primary (1°) immunoprecipitation of ^{35}S-labeled FL5.12Bcl-2HA–Bad#4 with 6C8 (lane 2) and secondary (2°) immunoprecipitation of that supernatant with anti-Bad Ab (lane 3).

Abbreviation: kD, kilodaltons.

formed with ^{35}S-labeled cells solubilized in 0.2% Nonidet P-40 (NP-40), which permits dimers of the Bcl-2 family to remain intact. The MAb to human Bcl-2, 6C8, immunoprecipitated the expected 25 kDa Bcl-2 and its heterodimerizing partner Bax from FL5.12Bcl-2 cells (Figure 3A). Immunoprecipitation of lysates from FL5.12Bcl-2HA–Bad#4 with 6C8 yielded a 30 kDa species in addition to Bcl-2 and Bax. The 12CA5 MAb immunoprecipitated the same 30 kDa band, confirming its identity as HA–Bad (Figure 3A). 12CA5 also immunoprecipitated a 25 kDa band, which proved to be Bcl-2 by Western blot analysis (Figure 3B). The anti-Bad polyclonal Ab showed the same immunoprecipitation pattern as 12CA5 (data not shown). Therefore, we have demonstrated that Bad and Bcl-2 proteins coprecipitate. Consistent with the yeast two-hybrid data that predicted the absence of Bad–Bax dimers, no Bax band was immunoprecipitated with HA–Bad in the 12CA5 lane. This also demonstrates that Bcl-2, which is bound to HA–Bad, is not heterodimerized with Bax. Thus, there is no evidence for the presence of Bad–Bcl-2–Bax heterotrimers.

A parallel series of immunoprecipitations was performed on FL5.12Bcl-x_LHA–Bad clones using an anti-Bcl-x polyclonal Ab. In FL5.12Bcl-x_L cells, anti-Bcl-x Ab captured the 28 kDa Bcl-x_L protein and a 21 kDa protein that has been proven to be Bax (Figure 4A) (Sedlak and S. J. K., unpublished data). In FL5.12Bcl-x_LHA–Bad#7 lysates, anti-Bcl-x Ab precipitated Bcl-x_L and a 30 kDa species consistent with HA–Bad, the identity of which was confirmed on a Western blot using anti-Bad Ab (Figure 4C). Similarly, in addition to the 30 kDa HA–Bad, both 12CA5 MAb and anti-Bad Ab precipitated a 28 kDa species, which was confirmed to be Bcl-x_L on immunoblotting (Figure 4B). Thus, HA–Bad and Bcl-x_L proteins coprecipitate. Again, the Bcl-x_L molecules that are bound to Bad are not heterodimerized with Bax. Moreover, in the anti-Bcl-x immunoprecipitate

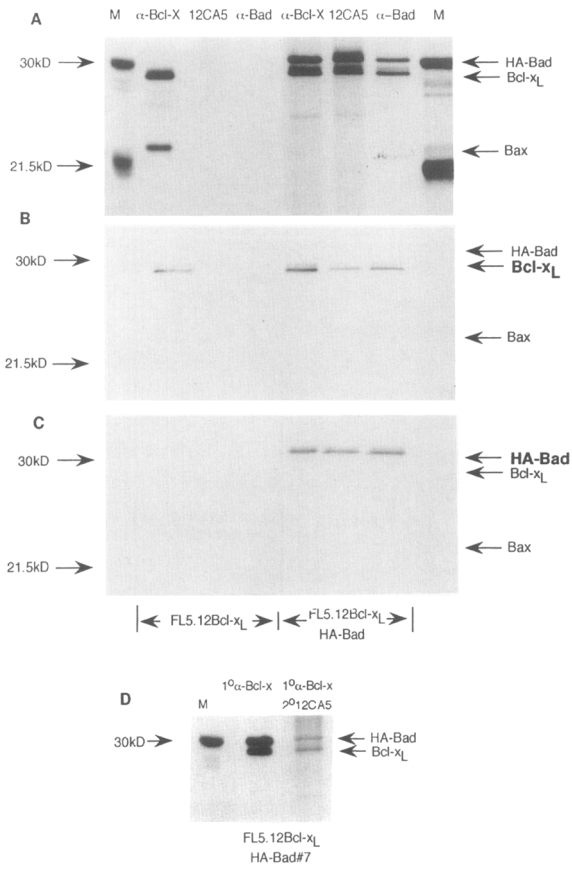

Figure 4. Coimmunoprecipitation of Bcl-x_L and Bad

(A) Primary immunoprecipitation of the ^{35}S-labeled FL5.12Bcl-x_L line (lanes 2, 3, 4) and one of the pSFFVHA–Bad transfected clones (lanes 5, 6, 7) using anti-Bcl-x Ab, 12CA5, and anti-Bad Ab. One third of each sample was loaded on a 12.5% SDS–PAGE gel and fluorographed.
(B) Western blotting of one third of each primary immunoprecipitate using biotinylated anti-Bcl-x Ab, developed with diaminobenzidine.
(C) Western blot of one third of each primary immunoprecipitate using biotinylated anti-Bad Ab, developed with diaminobenzidine.
(D) Shown here is primary (1°) immunoprecipitation of ^{35}S-labeled FL5.12Bcl-x_LHA–Bad#7 with anti-Bcl-x (lane 2) and secondary immunoprecipitation of that supernatant with 12CA5 (lane 3).
Abbreviation: M, marker lane.

of FL5.12Bcl-x_LHA–Bad#7, little or no Bax is present, indicating that nearly all of the Bcl-x_L is dimerized with HA–Bad (Figure 4A).

To assess further the extent to which HA–Bad had complexed with Bcl-2 or Bcl-x_L, secondary immunoprecipitations were performed on the supernatants of the aforementioned experiments. In FL5.12Bcl-2HA–Bad#4 cells, the intensity of Bcl-2 to HA–Bad bands in the primary 6C8 immunoprecipitates showed a ratio of ~2:1 by phosphorimager scanning. When the supernatant of this Bcl-2-depleted immunoprecipitate was reprecipitated with anti-Bad Ab, a reversed ratio of Bcl-2 to HA–Bad of ~1:3 was noted (see Figure 3C), indicating that excess Bad, not heterodimerized with Bcl-2, existed. However, a parallel experiment with FL5.12Bcl-x_LHA–Bad#7 cells revealed a Bcl-x_L to HA–Bad ratio of ~1:1 in primary anti-Bcl-x immunoprecipitates. When the supernatant of this Bcl-x_L–depleted immunoprecipitate was reprecipitated with anti-Bad Ab or 12CA5, only small amounts of residual Bcl-x_L and HA–Bad were detected at a ~1:1 ratio (Figure 4D), consistent with these proteins being complexed as heterodimers. In FL5.12Bcl-x_LHA–Bad#7 cells, nearly all the Bcl-x_L is heterodimerized with HA–Bad, and virtually none is heterodimerized with Bax (Figure 4A). In FL5.12Bcl-2HA–Bad#4 cells, despite an excess of HA–Bad, a substantial fraction of Bcl-2 is still heterodimerized with endogenous Bax (see Figure 3A). These data are consistent with a stronger association between Bcl-x_L and Bad than that between Bcl-2 and Bad.

Bad Counters the Death Inhibition by Bcl-x_L, but Not That by Bcl-2

Both Bcl-2 and Bcl-x_L function as death repressors, extending the viability of FL5.12 cells following IL-3 withdrawal (Hockenbery et al., 1990; Boise et al., 1993). The viability curves of the six FL5.12HA–Bad clones deprived of IL-3 were not convincingly different from those of FL5.12Neo control lines (data not shown), indicating that overexpressed Bad by itself did not affect apoptosis in this cell line. In addition, the survival curves for two independent Bcl-2- and Bad-overexpressing clones, FL5.12Bcl-2HA–Bad#4 and -#15, did not vary significantly from that of FL5.12Bcl-2 cells (see Figure 2B), showing that Bad had no substantial effect on the death repressor function of Bcl-2. In contrast, the viability of clones coexpressing Bcl-x_L and Bad proved significantly different from that of FL5.12Bcl-x_L cells. FL5.12Bcl-x_LHA–Bad#7 clone expressed a high level of Bad and exhibited a viability that nearly reverted to the pattern of several FL5.12Neo control lines (see Figure 2B). FL5.12Bcl-x_LHA–Bad#8 expressed a low amount of Bad protein (Figure 5A) and showed a slightly diminished viability compared with FL5.12Bcl-x_L upon IL-3 withdrawal (see Figure 2B). Therefore, at similar levels of expression (see Figure 2A), Bad countered the death repressor activity of Bcl-x_L, but not that of Bcl-2, in this death assay. Immunoprecipitations using mouse thymocytes revealed an association of endogenous Bcl-x_L and Bad in vivo (data not shown), arguing that this interaction will be relevant in normal cells.

Bad Successfully Competes for Bcl-x_L, Resulting in Free Bax

The differential effectiveness of Bad in countering Bcl-x_L but not Bcl-2 activity may be explained by the observation that more of the available Bad is bound to Bcl-x_L than to Bcl-2 (see Figures 3 and 4). Since heterotrimers do not appear to exist, this implies that the amount of Bax that is "unbound", that is, not heterodimerized to Bcl-x_L or Bcl-2, would be affected by the presence of Bad. To test this hypothesis, the amount of Bax not involved in Bcl-x_L–Bax or Bcl-2–Bax heterodimers was determined. Sequential immunoprecipitations were performed in the following manner: primary immunoprecipitations were performed on ^{35}S-labeled FL5.12Bcl-x_LHA–Bad and FL5.12Bcl-2HA–Bad clones in 0.2% NP-40 using either anti-Bcl-x Ab or 6C8, respectively (Figure 5A); the supernatants were cleared a second time with these antibodies to remove

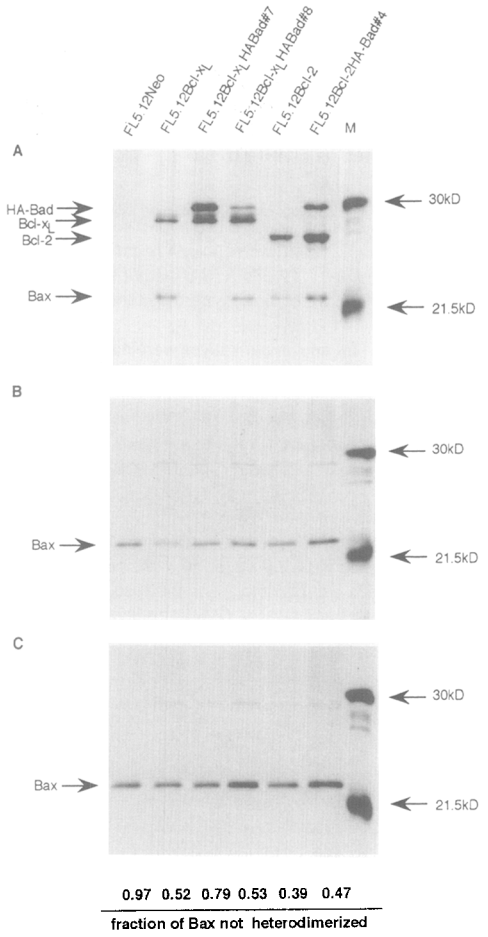

Figure 5. The Amount of Bax Not Found in Heterodimers Correlates with Cell Death

Sequential immunoprecipitations were performed on the clones indicated above the lanes.

(A) Using half of the ^{35}S-labeled cells from each clone, anti-Bcl-x was incubated with 0.2% NP-40 lysates of clones expressing Bcl-x_L, and 6C8 was incubated with those expressing human Bcl-2. Primary immunoprecipitates were separated on 12.5% SDS–PAGE gels and fluorographed.

(B) Immunoprecipitation of Bax from supernatants from the cells in (A). The supernatants from (A) were cleared a second time with the same Ab to remove all Bcl-x_L and Bcl-2 complexes. These doubly cleared supernatants were incubated with the 4D2 anti-Bax MAb and were electrophoresed on 12.5% SDS–PAGE gels to quantitate the amount of Bax not complexed with either Bcl-x_L or Bcl-2.

(C) The other half of the ^{35}S-labeled cells was lysed in radioimmunoprecipitation assay buffer and was immunoprecipitated with 4D2 to quantitate the total amount of Bax. Bands were quantitated by a phosphorimager. The fraction of Bax not in heterodimers with Bcl-x_L or Bcl-2 was determined in three experiments, and the average value is shown.

Abbreviations: kD, kilodaltons; M, marker lane.

all Bcl-x_L or Bcl-2 complexes; finally, these heterodimer-depleted supernatants were immunoprecipitated with the MAb anti-Bax, 4D2 (Figure 5B). One half of the original ^{35}S-labeled cells were lysed in radioimmunoprecipitation assay buffer, which disrupts dimers of this family, and were immunoprecipitated with 4D2 to establish the total amount of cellular Bax in each preparation (Figure 5C). The amount of Bax present in each precipitation was quantitated by phosphorimager scanning. This experiment was performed three times, and the average value for the fraction of Bax not found in heterodimers is presented (Figure 5).

In FL5.12Neo control cells, most of the Bax (97%), presumably present as homodimers, was not heterodimerized, as shown previously (Oltvai et al., 1993). The primary immunoprecipitation with 6C8 and anti-Bcl-x confirmed the persistence of Bcl-2–Bax heterodimers in FL5.12Bcl-2HA–Bad#4 and Bcl-x_L–Bax heterodimers in FL5.12Bcl-x_LHA–Bad#8 but not in FL5.12Bcl-x_LHA–Bad#7 (Figure 5A). Reciprocally, the amount of Bad in Bcl-x_L–Bad heterodimers in clone #7 is greater than the amount of Bad in Bcl-2–Bad heterodimers in clone #4 (Figure 5A). This resulted in a marked difference in the fraction of Bax not present in heterodimers. In FL5.12Bcl-x_L and FL5.12Bcl-2 cells that are protected from apoptosis, half or less of the total Bax was not heterodimerized (52% and 39%, respectively). In FL5.12Bcl-x_LHA–Bad#7 cells that have regained susceptibility to death, as much as 79% of the total Bax was not complexed to Bcl-x_L. In contrast, in FL5.12Bcl-x_LHA–Bad#8 and FL5.12Bcl-2HA–Bad#4, which still retained considerable protection, the percent of "free" Bax, that not found in heterodimers with Bcl-x_L or Bcl-2, was 53% and 47%, respectively (Figure 5).

Discussion

Both yeast two-hybrid and λ expression cloning experiments identified the same novel Bcl-2 family member, Bad. This protein qualifies for Bcl-2 family membership in that it possesses the key amino acid motifs of BH1 and BH2 domains, and a small region of Bad containing BH1 and BH2 binds Bcl-2. However, outside BH1 and BH2, Bad is quite distinct from other members. Moreover, Bad lacks the classic C-terminal signal–anchor sequence responsible for the integral membrane position of other family members. Bcl-2 has been localized to the mitochondrial membrane, endoplasmic reticulum, and nuclear membrane (Hockenbery et al., 1990; Monaghan et al., 1992; de Jong et al., 1994), and Bcl-x_L has been immunolocalized to the outer mitochondrial membrane (González-García et al., 1994). In vitro targeting studies have demonstrated that the C-terminal tail of Bcl-2 serves as a signal–anchor sequence capable of integrating into a variety of membranes (Nguyen et al., 1993). Protease digestions confirm that the majority, if not all, of Bcl-2 has its N-terminus facing the cytosol. Thus, Bcl-2 or Bcl-x_L could be expected to interact with a predicted cytosolic protein such as Bad. It is possible that Bad could communicate with other cytosolic proteins connecting the genetic pathway of cell death with other cellular processes.

Yeast two-hybrid analysis indicated that Bad would interact with Bcl-x_L as well as with Bcl-2. Immunoprecipitations in mammalian cells confirmed this prediction. The data favor a model of simple dimers in which Bad competes with Bax for association with Bcl-2 or Bcl-x_L. There was no evidence for the existence of any heterotrimers. The binding of Bad appeared to preclude the binding of Bax to either Bcl-x_L or Bcl-2. In cells with abundant Bcl-x_L–Bad heterodimers, no Bcl-x_L–Bax heterodimers were pres-

ent. In Bcl-2-expressing cells, less Bcl-2 is complexed with Bad, and Bcl-2–Bax heterodimers were still present. This suggested that Bad has a higher affinity for Bcl-x_L than for Bcl-2, although unanticipated differences in the subcellular availability of each protein could also affect these findings.

Cell death assays corroborated the significance of Bad heterodimerizations. Bad itself probably does not function as a singular, downstream death effector molecule because, when expressed by itself, it had no effect upon the death course of FL5.12 cells deprived of IL-3. FL5.12 cells express little Bcl-2, no detectable Bcl-x_L, and abundant Bax protein. In these cells, Bad countered the death inhibition by Bcl-x_L much more than it countered that by Bcl-2. This correlated with the proclivity of Bad to heterodimerize with Bcl-x_L more than it heterodimerized with Bcl-2.

Recently, Bcl-x_L has been shown to heterodimerize with Bax in mammalian cells as well as in yeast two-hybrid cells. A WAR for WGR substitution of the critical glycine in the BH1 domain was generated for Bcl-x_L. Parallel to this mutation in Bcl-2, this Bcl-x_L ml-3 protein no longer heterodimerized with Bax and also lost its death repressor activity (Sedlak and S. J. K., unpublished data). This provides further evidence for a model in which both Bcl-x_L and Bcl-2 must dimerize with Bax to repress death. The data with Bad further support this thesis and adds a third layer of complexity. Overall, the data argue for a simple competition in which Bad binds Bcl-x_L, displacing Bax into homodimers. Susceptibility to cell death is best correlated with the percent of Bax in heterodimers versus that in homodimers. If roughly half of Bax is complexed in heterodimers, FL5.12 cells will be protected from death. Formally, it is not certain whether Bax–Bax homodimers are the active moiety that might communicate with a death effector pathway or whether each Bcl-2–Bax or Bcl-x_L–Bax heterodimer is the active component.

The Bcl-x_L, Bax, and Bad system is reminiscent of the Myc, Max, and Mad regulatory network of basic helix-loop-helix transcription factors (Amati et al., 1993; Ayer et al., 1993). Myc–Max and Mad–Max heterodimers display positive and negative regulation of transcription, respectively. Myc and Mad both compete for binding to Max, and their relative levels determine the dominant heterodimer. Mad displays several characteristics parallel to Bad in that Mad is also a modulatory protein that does not regulate transcription by itself, but functions by sequestering Max in complexes that prevent it from binding Myc to activate transcription. In a parallel fashion, Bad is a regulator of apoptosis that appears to function by sequestering Bcl-x_L and by preventing Bcl-x_L from heterodimerizing with Bax.

The susceptibility to cell death is dictated by a complex set point determined by the relative levels and interactions of Bcl-x_L, Bcl-2, Bax, and Bad proteins. The presence of the novel protein, Bad, is one regulator that can determine whether the Bcl-2 or the Bcl-x_L molecule will be effective in repressing apoptosis.

Experimental Procedures

Plasmid Constructions

Standard PCR reactions were used to construct yeast fusion plasmids. The amino acids in the C-terminal transmembrane segments were deleted as follows: 21 amino acids were deleted from muBcl-2, 18 from muBax, 19 from huBcl-x_L, 19 from huBcl-x_S, 21 from muMcl-1, and 21 from muA1.

The expression plasmid pSFFVHA–Bad was constructed by using PCR primers, which incorporated the 9 codons of the HA epitope (Kolodziej and Young, 1991) onto the 5′ end of *bad*, and it was ligated into pSFFV vector at the EcoRI site.

Strains and Cell Lines

The yeast strains used in the yeast two-hybrid screening were PCY2 (Chevray and Nathans, 1992) and Y190 (*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3::GAL–lacZ LYS::GAL–HIS3 cyh*^{r}). Yeast transformations were done using the standard lithium acetate procedure.

The FL5.12 cell line and its derivatives were cultured as previously described (McKearn et al., 1985).

Yeast Two-Hybrid Screening

PCY2 harboring the GAL4 DNA-binding domain Bcl-2 plasmid was transformed with 4 μg of an oligo(dT)-primed mouse 14.5 day embryo fusion cDNA library constructed in the GAL4-activating domain vector. Transformants were plated on −Leu −Trp medium and were screened by the filter X-Gal assay (Chevray and Nathans, 1992). Blue colonies cured of the bait plasmid were mated to Y190 carrying either vector alone or nonspecific bait plasmids, and the rescued cDNA plasmids were cotransformed back into PCY2 with irrelevant bait plasmids so as to identify those cDNA isolates that were specifically positive with Bcl-2.

Expression Cloning

Bacterially produced GST–HMK–Bcl-2ΔC21 was purified, labeled, and used to screen phage filters in 0.05% NP-40 and 1 mM DTT, according to the protocol of Blanar and Rutter (1992).

cDNA Screening

A *bad* coding region probe was used to screen 1 × 10^6 plaques each of a newborn mouse brain and an adult mouse thymus λZAPII cDNA library. Standard phage screening techniques were employed.

Transfections

FL5.12 cells were electroporated at 200 V and 900 μF. Later (48 hr), the cells were plated at limiting dilution in medium containing 1 mg/ml G418 in 96-well microtiter dishes. Single-cell origin clones were picked 10–14 days later.

Antibodies

6C8 is a human Bcl-2-specific hamster MAb (Hockenbery et al., 1990). 12CA5 is a murine MAb against influenza virus HA protein epitope. 4D2 is a hamster MAb against murine Bax (Sedlak and S. J. K., unpublished data). Anti-Bcl-x Ab is a polyclonal Ab against Bcl-x. The rabbit polyclonal anti-Bad Ab was raised against bacterially produced protein and was protein A purified. For immunoprecipitations, 6C8 was used at a final concentration of 20 μg/ml, 12CA5 was used at 40 μg/ml, anti-Bcl-x Ab was used at 10 μl per 1 × 10^7 cells. 4D2 and anti-Bad Ab were titrated to be maximally efficient at 48 μg/ml and 60 μg/ml.

Western Blotting Analysis

Cells were lysed in 100 mM Tris (pH 7.5), 75 mM NaCl, 1% Triton X-100, 1% PMSF, and 1% aprotinin. Standard Western blotting procedures were employed. Abs were incubated for 1 hr at room temperature. 12CA5 MAb supernatant was used at 1:50 dilution, anti-Bcl-x Ab was used at 1:1000, anti-Bad Ab was used at 1:1000, and 6C8 was used at 1:100. Biotinylated secondary goat Ab (Caltag) was used at 1:500, and horseradish peroxidase–conjugated streptavidin (Zymed) was used at 1:1000 when developed with diaminobenzidine and at 1:20,000 when developed with ECL.

Immunoprecipitations

Each immunoprecipitation used 1 × 10^7 cells labeled in methionine-free Dulbecco's modified Eagle's medium, 10% dialyzed fetal calf serum, 10% WEHI-3B supernatant, and 100 μCi of Tran^{35}S-label for 12 hr. Subsequent steps were carried out as previously described (Oltvai et al., 1993). ^{35}S-labeled bands were quantitated by phosphorimager scanning.

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