

Drosophila Pro-apoptotic Bcl-2/Bax Homologue Reveals Evolutionary Conservation of Cell Death Mechanisms*

Received for publication, April 4, 2000, and in revised form, May 9, 2000
Published, JBC Papers in Press, May 12, 2000, DOI 10.1074/jbc.M002846200

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Genetic analysis of programmed cell death in *Drosophila* reveals many similarities with mammals. Herefore, a missing link in the fly has been the absence of any Bcl-2/Bax family members, proteins that function in mammals as regulators of mitochondrial cytochrome *c* release. A *Drosophila* homologue of the human killer protein Bok (DBok) was identified. The predicted structure of DBok is similar to pore-forming Bcl-2/Bax family members. DBok induces apoptosis in insect and human cells, which is suppressible by anti-apoptotic human Bcl-2 family proteins. A caspase inhibitor suppressed DBok-induced apoptosis but did not prevent DBok-induced cell death. Moreover, DBok targets mitochondria and triggers cytochrome *c* release through a caspase-independent mechanism. These characteristics of DBok reveal evolutionary conservation of cell death mechanisms in flies and humans.

Genetic analysis of programmed cell death in *Drosophila* reveals many similarities with mammals (reviewed in Ref. 1). For example, caspase family cell death proteases, inhibitor of apoptosis protein family caspase inhibitors, and CED-4 family caspase activators have been described in both flies and humans. However, a missing component of the cell death machinery in the fly has been Bcl-2/Bax family proteins.

In mammals, Bcl-2/Bax family proteins function as regulators of mitochondrial cytochrome *c* release (2). Pro-apoptotic Bcl-2 family proteins such as Bax and Bid induce release of cytochrome *c* from mitochondria (3–5), perhaps in part due to their structural similarity with pore-forming bacterial toxins (6–9). In contrast, anti-apoptotic Bcl-2 family proteins prevent cytochrome *c* release, and maintain outer mitochondrial membrane impermeability to proteins (2).

After release from mitochondria, cytochrome *c* binds a cytosolic activator of caspases in mammals (Apaf-1) and in flies (Dapaf-1/Dark/HAC1) (reviewed in Refs. 1 and 10). Apaf-1 is normally inactive, but cytochrome *c*-induced oligomerization of Apaf-1 allows it to bind and activate pro-caspase-9, thus initi-

ating a cascade of proteolysis that culminates in apoptosis (11, 12).

Sequencing of the genome of *Drosophila melanogaster* is near completion, permitting a search for Bcl-2/Bax homologues. We describe here a pro-apoptotic Bcl-2 family member from *Drosophila*, which has functional characteristics highly similar to its human counterparts, such as Bax, Bak, and Bok (Mtd). The findings have important implications for understanding the evolution of cell death mechanisms, and suggest some striking differences in the way Bcl-2/Bax family proteins function in mammals and flies compared with the nematode *Caenorhabditis elegans* in which much of the original genetic analysis of programmed cell death was performed.

MATERIALS AND METHODS

Cloning of DBok—Using the human Bcl-2 sequence to perform TBLASTN searches (13), a fragment of DBok was found in the *Drosophila* Expressed Sequence Tag Data Base (accession no. AI513093) on May 13, 1999. DNA was prepared from a *Drosophila* embryo cDNA library, and amplified by PCR¹ using DBok-specific primers. For this procedure, primers were designed according to the Sp6 and T7 promoter sequences within the cDNA library vector and the sequence of the DBok genomic fragment found in the data base: 5'-ATTAGGTGACAC-TATAG-3' and 5'-GGCAAATATCGAGATTATCTTGC-3'; 5'-TAATAC-GACTCACTATAGGGA-3' and 5'-GAGCGGATGCACCCGCG-3' were used for PCR to clone the 5' and 3' end of DBok, respectively. PCR was performed for 35 cycles using 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min. Amplified fragments were cloned into pCR-BluntII-TOPO vector (Invitrogen) and sequenced (GenBank[®] accession number AF228044). The predicted start codon for the DBok open reading frame (ORF) resides in a favorable context for translation with adenosine at the –3 position and cytosine at the –4 position (14) but is preceded by two additional upstream in-frame AUGs, which are in unfavorable contexts with either uracil at the –3 position or guanine at the –4 position (uracil at the –3 position and guanine at the –4 position account for 8% and 12% of ORFs in *Drosophila*, respectively) (14, 15). Upstream stop codons were found in all three reading frames. An in-frame upstream possible start codon was rejected as a likely start site because of unfavorable Kozak context but cannot be excluded (16, 17). The DBok sequence is contained within the N-terminal part of the gene CG12397 (transcript CT26692) in the *Celera* fly genomic data base (18).

Plasmid Construction—A 645-base pair DBok-encoding cDNA fragment encompassing the complete ORF of DBok was PCR-amplified and subcloned into the *EcoRI-XhoI* sites in the plasmids pCDNA3myc, pEGFY (Clontech), and pGilda or into the *EcoRI-SalI* sites of pSPMC vector. DBokΔBH3, a BH3 deletion mutant lacking residues MGEEELER was constructed by a two-step PCR method (19), using the primer GTGTACACCCGCGGGTGCATGTTCAGTGCCGGGAAAAC-TC, and subcloned into pCDNAs-Myc. Plasmids encoding human Bcl-X_L and Mcl-1 have been described (20).

Cell Culture, Transfection, and Apoptosis Assays—HEK 293 cells

* This work was supported by National Institutes of Health Grant GM 60554. 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.

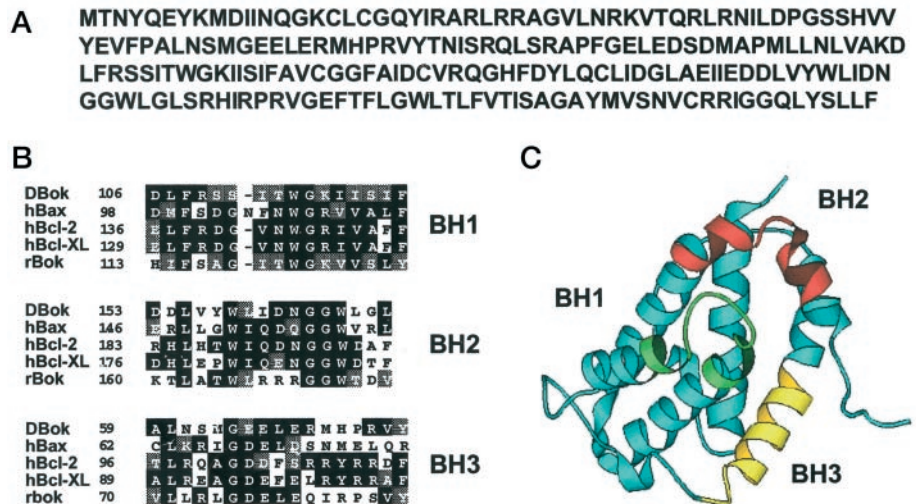
The nucleotide sequence(s) reported in this paper has been submitted to GenBank[™]/EBI Data Bank with the accession number(s) AF228044.

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¹ The abbreviations used are: PCR, polymerase chain reaction; GFP, green fluorescent protein; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; DAPI, 4',6-diamidino-2-phenylindole.

FIG. 1. Predicted sequence and structure of the DBok protein. *A*, the predicted amino acid sequence of DBok is presented. *B*, an alignment of the BH1, BH2, and BH3 domains of DBok with other Bcl-2 family proteins is shown, representing identical and similar residues in *black* and *gray boxes*, respectively. *C*, a model of the predicted DBok protein structure, as derived using the structure of Bcl-X_L and employing the MODELLER program, is presented with BH1 in *green*, BH2 in *red*, and BH3 in *yellow*. The N terminus of the protein begins at *top* and C terminus ends at the *right side* of the figure.



were maintained in Dulbecco's modified Eagle's medium (Irvine Scientific) supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and antibiotics. 293 cells (5×10^5) in six-well dishes were co-transfected using Superfect (Qiagen) with 0.1 μ g of green fluorescence protein (GFP) marker plasmid pEGFP (CLONTECH), and 2 μ g of either pcDNA3myc-DBok or pcDNAmyc-DBok Δ BH3. Alternatively, cells were co-transfected with 2 μ g of pcDNAmyc-DBok and 2 μ g of pcDNA-Bcl-X_L or pcDNA-Mcl-1. Both floating and adherent cells were recovered 24–36 h after transfection and pooled, and the percentage of GFP-positive cells with nuclear apoptotic morphology was determined by staining with 0.1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) (mean \pm S.D.; $n = 3$). Alternatively, cell viability was assessed by trypan blue dye exclusion using unfixed cells. In some cases, lysate were prepared from transfected cells, normalized for total protein content, and analyzed by SDS-PAGE/immunoblotting using antibodies specific for Myc tag (Santa Cruz), Bcl-X_L, or Mcl-1, with enhanced chemiluminescence (ECL) detection (Amersham Pharmacia Biotech) (21).

Insect Sf9 cells were maintained at 27 °C in TMN-FH insect medium (Sigma) supplemented with 10% fetal bovine serum. Sf9 cells (10^6) were transfected using 6 μ l of Fugene 6 (Roche Molecular Biochemicals) with 1 μ g of ppsp-EGFP marker plasmid and 1 μ g of ppsp-DBok or ppsp-DBok Δ BH3. At 1 day after transfection, the cells were heat-shocked at 42 °C for 30 min and allowed to recover at 27 °C for 30 min. The heat shock treatment was repeated twice. Cells were recovered at 24 h after heat shock and pooled, and the percentage of GFP-positive cells with nuclear apoptotic morphology was determined by staining with 0.1 μ g/ml DAPI (mean \pm S.D.; $n = 3$).

Fluorescence Microscopy—COS-7 cells were transfected with 5 μ g of pEGFP or pEGFP-DBok. Photomicrographs were taken 20 h after transfection using a fluorescence microscope.

Subcellular Fractionation—For cytochrome *c* release assays, HEK293 cells (2×10^6 cells/10-cm dish) were transfected with 15 μ g of pcDNA3 (control) or pcDNA3-Myc-DBok. At 18 h after transfection, cells were collected for subcellular fractionation as described (3), and the relative proportions of cytochrome *c* in cytosolic and mitochondrial compartments were assessed by SDS-PAGE/immunoblotting using antibodies specific for cytochrome *c* (PharMingen), mitochondrial F1 α (Molecular Probes), Hsp60 (Santa Cruz), and β -tubulin (PharMingen). In some cases, cells were cultured with 100 μ M benzoyl-Val-Ala-Asp-fluoromethylketone (Bachem) beginning at 1 h after transfection. For assessing the intracellular location of DBok, transfected cells were lysed by homogenization in hypotonic detergent-free buffer (22) and nuclei were discarded by centrifugation at $500 \times g$ for 5 min. Post-nuclear lysates were then centrifuged at $10,000 \times g$ for 20 min to obtain a heavy membrane fraction, followed by $150,000 \times g$ for 1 h to obtain light membrane (pellet) and cytosolic (supernatant) fractions. Fractions were normalized for cell equivalents and analyzed by immunoblotting.

Molecular Modeling—A model of the predicted DBok protein structure was generated using the MODELLER program, essentially as described (7), based on the structure of Bcl-X_L (23).

RESULTS

Using the human Bcl-2 sequence for data base searches, we identified an expressed sequence tag clone (AI513093) and

subsequently cloned cDNAs encoding a 214-amino acid protein, which contains regions sharing extensive amino acid sequence homology with the BH1, BH2, and BH3 domains of other Bcl-2 family proteins (Fig. 1). Overall, the sequence of this protein was most similar to rat Bok, a pro-apoptotic Bcl-2 family member, with 30% sequence identity (52% similarity). The homology of DBok to other Bcl-2 family proteins was further analyzed by modeling the protein on the structure of human Bcl-X_L, confirming the prediction that DBok adopts a highly similar fold (Fig. 1C) of an irregular α -helical bundle, resembling the pore-forming domains of bacterial toxins. In particular, six α -helices are predicted, including a hairpin pair of largely hydrophobic α -helices in the center of the molecule, surrounded by a shell of four amphipathic α -helices.

To explore its effects on apoptosis, the DBok protein was overexpressed by transient transfection in Sf9 insect cells, along with a GFP marker. Alternatively, DBok was expressed as a GFP fusion protein, providing a convenient means of verifying expression. Staining of the fixed cells with a DNA-binding fluorochrome (DAPI) 1 day later revealed the presence of multiple apoptotic cells (with condensed chromatin, fragmented nuclei, and rounded, shrunken cell bodies) in cultures of DBok- but not control plasmid-transfected Sf9 cells (Fig. 2A). DBok also induced apoptosis of mammalian cells, such as HEK293, HT1080, and COS-7 (Fig. 2B and data not shown), implying evolutionary conservation of its cytotoxic function. DBok-induced apoptosis was consistently suppressed by co-expressing the anti-apoptotic Bcl-2 family protein Mcl-1 (Fig. 2C). Immunoblot analysis confirmed that DBok protein was still produced, and indeed was present at higher levels when cells were co-transfected with Mcl-1, suggesting that the cytoprotective effects of this anti-apoptotic Bcl-2 family protein allows the DBok protein to accumulate to higher levels. Bcl-X_L also partially inhibited DBok-induced apoptosis but was far more variable in its effects, inhibiting DBok-induced apoptosis by 0–50% (mean = 19%; $n = 3$). The BH3 domain of many pro-apoptotic Bcl-2 family proteins is required for dimerization with anti-apoptotic proteins such as Bcl-2 and Bcl-X_L and for induction of cell death (24, 25). However, similar to mammalian Bok (26), deletion of the BH3 domain of DBok did not interfere with apoptosis induction by DBok (Fig. 2D), indicating a BH3-independent mechanism.

Based on fluorescence UV microscopy analysis of cells transfected with a plasmid producing GFP-DBok fusion protein, the DBok protein was determined to be cytosolic, localizing to intracellular organelles in a pattern typical of Bcl-2 family

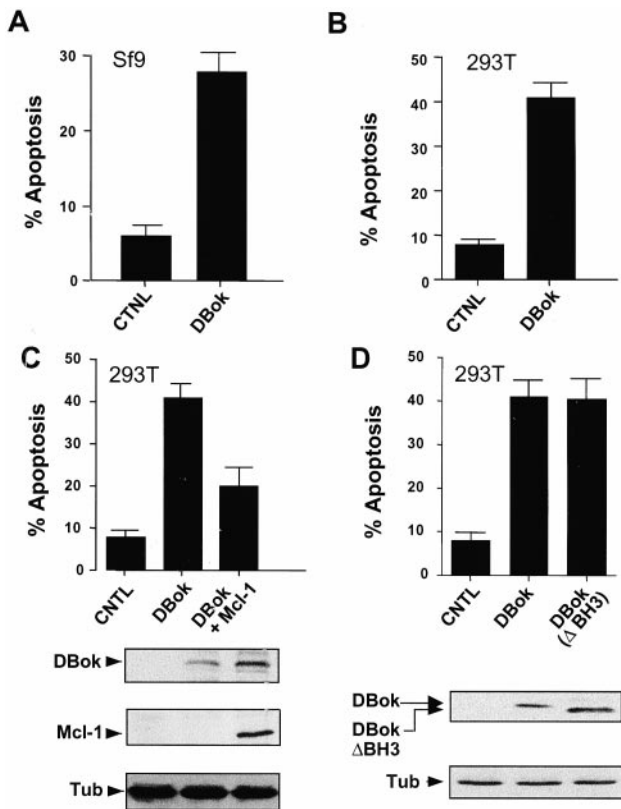


FIG. 2. DBok induces apoptosis in insect and mammalian cells.

A plasmid encoding either Myc-tagged DBok in pcDNA3 or control pcDNA3 (2 μ g) was transfected into Sf9 insect cells (A) and human epithelial kidney 293T cells (B) using cationic lipid reagents. A GFP-encoding plasmid (0.1 μ g) was included in all transfections revealing >50% Sf9 and >90% 293T cell transfection efficiency. After 20 h, both floating and adherent (after trypsinization) cells were pooled, fixed, and stained with DAPI (29). The percentage of GFP-positive cells with fragmented nuclei or condensed chromatin (apoptotic) was determined (mean \pm S.D.; $n = 3$). Similar results were obtained by expressing GFP-DBok protein (data not shown), which also verified protein expression. C, 293T cells were transiently co-transfected with 2 μ g of either pcDNA3 or pcDNA3-MycDBok plasmid together with 2 μ g of plasmid encoding Myc-tagged Mcl-1 or control plasmid. Cells were collected 20 h later and assayed for apoptosis by DAPI staining (mean \pm S.D.; $n = 3$). For immunoblotting (bottom), 293T cell extracts were prepared from an aliquot of the same transfected cells, normalized for total protein content (25 μ g), and subjected to SDS-PAGE/immunoblot analysis in which the blot was sequentially probed with antibodies recognizing Myc tag, Mcl-1, or β -tubulin (loading control) (29). D, 293T cells were transfected with 2 μ g of pcDNAmyc, pcDNAmyc-DBok, or pcDNAmyc-DBok Δ BH3. One day later, apoptosis (top) and protein expression (bottom) were measured as described above. Data are representative of multiple experiments.

proteins (Fig. 3A). Subcellular fractionation studies confirmed that DBok was associated at least in part with mitochondria-containing heavy-membrane fractions (Fig. 3B), despite absence of the C-terminal transmembrane domain, which is commonly found in many other Bcl-2 family proteins.

The mammalian pro-apoptotic Bcl-2 family members, which share structural similarity with pore-forming proteins (Bax, Bak, and Bid), induce release of cytochrome *c* from mitochondria (2). Similarly, in transfection experiments, expression of DBok in 293T cells induced an increase in cytosolic and a concomitant decrease in mitochondrial cytochrome *c* (Fig. 3B). Moreover, similar to mammalian Bax (3), release of cytochrome *c* was caspase-independent, as evidenced by failure of a broad spectrum caspase inhibitor, zVAD-fmk, to suppress it (Fig. 3B). Under the same conditions, however, zVAD-fmk suppressed DBok-induced caspase activation (determined by measuring cleavage of the caspase substrate acetyl-Asp-Glu-Val-Asp-ami-

nomethyl-coumarin (Ac-DEVD-AFC) (data not shown) and apoptosis, as determined by DAPI staining of fixed cells (Fig. 3C). Thus, zVAD-fmk effectively blocked the caspase activation that occurs downstream of cytochrome *c* release in DBok-expressing cells. However, analogous to previous reports for mammalian Bax (27), DBok-induced non-apoptotic cell death (as determined by failure of cells to exclude trypan blue dye) in the presence of caspase inhibitor, presumably due to the deleterious consequences of cytochrome *c* release on mitochondrial metabolic function. In contrast to zVAD-fmk, anti-apoptotic Mcl-1 protein suppressed DBok-induced cytochrome *c* release (data not shown), as well as DBok-induced apoptosis and cell death (Fig. 3C). Taken together, these findings suggest that DBok induces cell death through mechanisms similar to those employed by its mammalian counterparts.

DISCUSSION

Genetically tractable lower organisms have proven extremely insightful for delineating evolutionarily conserved mechanisms of programmed cell death. Though much of the genetics of programmed cell death was initially elucidated in the worm *C. elegans*, the fly *D. melanogaster* has provided additional apoptosis regulators not found in nematodes (1). Heretofore, the absence of any Bcl-2/Bax homologues left a void in the fly pathways for apoptosis relative to other organisms. The discovery of a Bcl-2/Bax homologue in *Drosophila* now at least partly fills that gap. However, still missing, is an anti-apoptotic Bcl-2 homologue in flies. In this regard, an additional gene with strong sequence homology to Bcl-2/Bax family proteins is present in the *Drosophila* genome (accession no. AC007473). After submission, additional reports of a fly Bcl-2 homologue (Drob-1; DeBcl) appeared, which correspond to the same gene described here (16, 17). However, these reports predict a longer protein, containing an additional N-terminal 86 amino acids not included in the DBok protein. Although the open reading frame we identified through DNA sequencing could encode a longer protein, we selected a downstream AUG as the candidate start codon based on its far more favorable Kozak context. The determination of which of these two possible isoforms of the DBok protein is more prevalent in flies therefore must await N-terminal sequencing or mass spectrometry analysis of the endogenous protein.

The DBok protein is an apoptosis-inducing member of the Bcl-2/Bax family. Two categories of pro-apoptotic Bcl-2 family proteins exist: (a) proteins that share structural homology with pore-forming bacterial toxins such as diphtheria toxin and the colicins, and (b) proteins that have in common only the presence of an amphipathic α -helical BH3 dimerization domain (24, 25). Homology modeling of the DBok sequence strongly suggests that it belongs to the pore-forming group of pro-apoptotic Bcl-2 family proteins, along with Bax, Bak, Bok/Mtd, and Bid. The ability of the DBok Δ BH3 mutant to induce apoptosis further supports this hypothesis. It should be noted, however, that Colussi *et al.* (17) reported a requirement for the BH3 domain of DBok for induction of apoptosis, suggesting that this domain can make important contributions to its death-inducing function in at least some circumstances.

Two opposing models for explaining the mechanisms of Bcl-2 family proteins have been proposed (2, 10). Studies of programmed cell death in *C. elegans* indicate that the anti-apoptotic Bcl-2 homologue CED-9 functions by binding and suppressing the Apaf-1 homologue CED-4, thus preventing caspase activation. Pro-apoptotic EGL-1 protein (a member of the "BH3 only" group of pro-apoptotic Bcl-2/Bax family proteins) in worms dimerizes with CED-9 via its BH3 domain and triggers CED-4 release from CED-9. The cytotoxic effect of EGL-1 is entirely dependent on downstream caspases (28). In

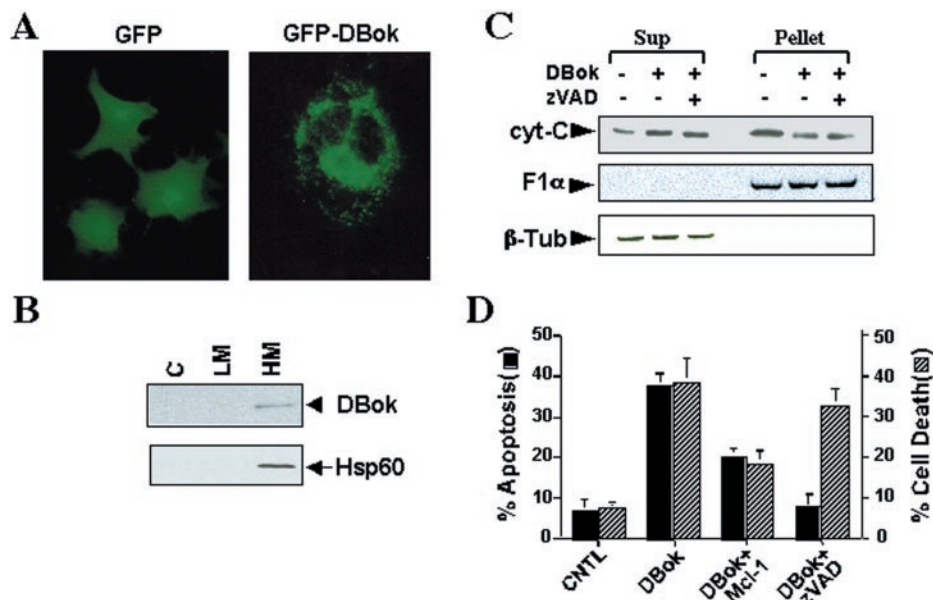


FIG. 3. DBok localizes to intracellular membranes, induces cytochrome *c* release, and kills cells through a caspase-independent mechanism. A, COS-7 cells were transfected with pEGFP-N2 plasmids encoding GFP or GFP-DBok fusion protein and viewed 1 day later by fluorescence microscopy, revealing association of DBok with cytosolic organelles. B, 293T cells expressing Myc-DBok were lysed to obtain a post-nuclear supernatant, which was further fractionated to yield heavy membrane (HM), light membrane (LM), and cytosolic fractions (22). Fractions were normalized for cell equivalents and analyzed by SDS-PAGE/immunoblotting using anti-Myc epitope tag antibody (top) or an antibody recognizing mitochondrial Hsp60 (bottom). C, DBok induces cytochrome *c* release. 293T cells were transfected with pcDNA3 or pcDNA3-Myc-DBok. In some cases, 100 μ M zVAD-fmk was added to cultures 1 h later. Cells were lysed in isotonic, detergent-free solution 1 day after transfection, and mitochondria-containing heavy membrane (pellet) and cytosolic (sup) fractions were prepared (30) and analyzed by SDS-PAGE/immunoblotting using anti-cytochrome *c*, as well as antibodies to a mitochondrial F_0F_1 -ATPase subunit ($F_1\alpha$) and cytosolic β -tubulin, which served as loading controls and verified proper fractionation. D, caspase inhibitor zVAD-fmk inhibits DBok-induced apoptosis without preventing cell death. 293T cells were co-transfected with 2 μ g of pcDNA3 or pcDNA3-Myc-DBok plasmid together with 2 μ g of control (CNTL) plasmid or plasmid encoding Myc-tagged Mcl-1. Some cultures received 100 μ M zVAD-fmk. Cells were collected 20 h later, and the percentages of apoptotic cells (black bars) and dead cells (shaded bars) were determined by DAPI staining of fixed cells and by trypan blue dye exclusion assay using unfixed cells, respectively (mean \pm S.E.; $n = 3$).

contrast to EGL-1, mammalian Bax can induce cell death and mitochondrial cytochrome *c* release, independent of binding Bcl-2 and independent of caspases (3, 27). DBok functions akin to mammalian Bax, inducing caspase-independent cytochrome *c* release and cell death. This evolutionary conservation of Bax/Bok protein function in mammals and flies is consistent with the cytochrome *c* inducibility of their Apaf-1 proteins (imparted by conserved WD repeat domains). By comparison, the CED-4 protein of *C. elegans* is constitutively active and has no requirement for cytochrome *c* (reviewed in Ref. 10). Although completely sequenced, the genome of *C. elegans* contains no discernible Bax/Bak/Bok homologues, implying that nematodes employ only a subset of the cell death mechanisms available to flies and mammals, which have developed methods for integrating mitochondria into their cell death machinery as devices for sensing stress and coupling it to caspase activation.

Acknowledgments—We thank R. Cornell for manuscript preparation and K. and R. Okano for plasmids.

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