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BID, BIM, and PUMA Are Essential for Activation of the BAX- and BAK-Dependent Cell Death Program

Decheng Ren^{1,*}, Ho-Chou Tu^{1,*}, Hyungjin Kim¹, Gary X. Wang¹, Gregory R. Bean¹, Osamu Takeuchi², John R. Jeffers³, Gerard P. Zambetti³, James J.-D. Hsieh¹, and Emily H.-Y. Cheng^{1,4,†,‡}

¹Molecular Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA

²Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan

³St. Jude Children's Research Hospital, Memphis, TN 38105, USA

⁴Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA

Abstract

Although the proteins BAX and BAK are required for initiation of apoptosis at the mitochondria, how BAX and BAK are activated remains unsettled. We provide in vivo evidence demonstrating an essential role of the proteins BID, BIM, and PUMA in activating BAX and BAK. *Bid*, *Bim*, and *Puma* triple-knockout mice showed the same developmental defects that are associated with deficiency of *Bax* and *Bak*, including persistent interdigital webs and imperforate vaginas. Genetic deletion of *Bid*, *Bim*, and *Puma* prevented the homo-oligomerization of BAX and BAK, and thereby cytochrome *c*-mediated activation of caspases in response to diverse death signals in neurons and T lymphocytes, despite the presence of other BH3-only molecules. Thus, many forms of apoptosis require direct activation of BAX and BAK at the mitochondria by a member of the BID, BIM, or PUMA family of proteins.

Mitochondria have key roles in mammalian apoptosis, a highly regulated genetic program of cell suicide (1-3). Multiple apoptotic signals release cytochrome *c* from the mitochondria to activate the Apaf-1 protein which activates caspases. The BCL-2 family of proteins integrates developmental and environmental cues to dictate the survival or death decision of cells by regulating the integrity of the mitochondrial outer membrane (MOM) (1, 4, 5). The multidomain proapoptotic proteins BAX and BAK mediate permeabilization of the MOM, whereas anti-apoptotic BCL-2, BCL-X_L, and MCL-1 proteins prevent cytochrome *c* efflux triggered by apoptotic stimuli. The third BCL-2 subfamily of proteins, the BH3-only molecules (BH3s) (that is, family members with only one BCL-2-homology domain), constitutes the largest BCL-2 subfamily with more than 10 members that promote apoptosis by either activating BAX and BAK directly or inactivating BCL-2, BCL-X_L, or MCL-1 (6-12). When apoptosis is initiated, BAK and BAX undergo conformational changes to form homo-oligomers that mediate cytochrome *c* efflux (6-9). Although BAX and BAK control the mitochondrial gateway to apoptosis, how BAX and BAK are activated, whether BAX

[‡]Correspondence: chenge1@mskcc.org.

^{*}These authors contributed equally to this work.

[†]Present address: Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA.

and BAK are activated directly or indirectly by BH3s, and the identity of the core repertoire of activators of BAX and BAK in various tissues remain unsettled. Two non-mutually exclusive models have been proposed (5, 13). The direct activation model states that the “activator” subgroup of BH3s, including truncated BID (tBID) and BIM can directly induce the conformational changes of BAX and BAK (6-12, 14-16). The indirect model proposes that activation of BAX and BAK occurs by default as long as all the anti-apoptotic BCL-2 proteins are neutralized by BH3s, based on the observation that BAX - or BAK-dependent apoptosis proceeds in the absence of BID and BIM (17). However, PUMA appears also to function as a direct activator of BAX and BAK. In vitro translated PUMA protein, but not the BH3 domain peptide of PUMA, can directly activate BAX- and BAK-dependent permeabilization of the MOM (11, 12, 18-20). The transmembrane domain was important for PUMA to induce cytochrome c efflux (fig. S1), which may explain why BH3 peptides are less active (11, 19). Thus, we studied *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} triple-knockout (TKO) mice to clarify how BAX and BAK are activated and whether BID, BIM, and PUMA represent the core repertoire of activators of BAX and BAK that function downstream of other BH3s.

Although the embryonic lethality caused by triple deficiency of *Bid*, *Bim*, and *Puma* appeared to be less severe than that in mice lacking *Bax* and *Bak* (table S1), the viable *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} mice displayed developmental defects very similar to those of *Bax*^{-/-}*Bak*^{-/-} animals, including persistent interdigital webs of skin on their feet and imperforate vaginas (21) (Fig. 1, A and B, and table S2). To determine whether deficiency of *Bid*, *Bim*, and *Puma* completely blocks the intrinsic apoptotic death as deficiency of *Bax* and *Bak* does, we examined ionizing radiation (IR)-induced apoptosis in cerebellar granule neurons (CGN). Because most of the *Bax*^{-/-}*Bak*^{-/-} mice die in embryogenesis, we used the Cre-LoxP conditional knockout strategy to delete *Bax* in neurons through a Nestin-Cre construct that is expressed in neuronal and glial cell precursors (22). No activated caspase-3 was detected in *Bax*^{f/f}*Bak*^{-/-}*Nestin-Cre*⁺ neurons (Fig. 1D). *Puma*-deficient neurons were less sensitive to IR than *Bax*-deficient neurons (Fig. 1E and fig. S2). Although deficiency of *Puma* greatly reduced the activation of caspase-3, deletion of *Bid*, *Bim*, and *Puma* was required to completely block caspase-mediated apoptosis (Fig. 1, E to H). Similar findings were also observed in IR-induced apoptosis in dentate gyrus. BAD and BMF proteins were detected in CGN (fig. S3), suggesting that BAD and BMF were unable to activate BAX- or BAK-dependent apoptosis in the absence of *Bid*, *Bim*, and *Puma*. These data indicate that activation of BAX and BAK in response to IR is fully dependent on BID, BIM, and PUMA.

To exclude the possibility that BID, BIM, and PUMA are required to activate BAX or BAK only in response to genotoxic stress, we investigated potassium-deprivation-induced apoptosis of cultured CGN. CGN from early postnatal mice can be cultured in medium containing a high concentration of potassium (25 mM) that provides depolarization-mediated survival. Deprivation of potassium (5 mM) induces BAX-dependent apoptosis that requires de novo protein synthesis (23). Consistent with the previous reports demonstrating the accumulation of BIM and PUMA proteins in potassium-deprived CGN (24, 25), deficiency of either *Bim* or *Puma* provided transient protection, whereas deficiency of both *Bim* and *Puma* conferred longterm protection against potassium-deprivation-induced apoptosis (Fig. 2, and figs. S4 and S5). Deletion of *Bid*, *Bim*, and *Puma* completely blocked apoptosis up to 3 days (Fig. 2, and figs. S4 and S5). These data indicate that BAX is not activated in the absence of *Bid*, *Bim*, and *Puma*, even though BAD and BMF are present (fig. S3) and abundance of HRK (also called DP5) is increased in these neurons (26).

One of the major phenotypes of *Bax*^{-/-}*Bak*^{-/-} mice is accumulation of lymphoid and myeloid cells as a consequence of defective apoptosis (21). *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} animals also exhibited enlarged thymi, splenomegaly, and lymphadenopathy (fig. S6). The role of PUMA as a direct activator of BAX and BAK is debated in part because of reports showing that

knockdown of *Puma* does not provide further inhibition of apoptosis in the absence of *Bid* and *Bim* (17) and because exogenous PUMA fails to induce BAX- and BAK-dependent mitochondrial permeabilization in the absence of *Bid* and *Bim* (27). To address whether endogenous PUMA can activate BAX- and BAK-dependent mitochondrial apoptosis independently of BID and BIM, we compared the apoptotic phenotypes of *Bid*^{-/-}*Bim*^{-/-} double knockout (DKO) mice to *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO mice. The TKO mice had more pronounced accumulation of hematopoietic cells in thymus, spleen, lymph nodes, and blood than DKO mice (figs. S6 to S8). Moreover, *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO T cells were more resistant than *Bid*^{-/-}*Bim*^{-/-} DKO cells to diverse intrinsic apoptotic signals, including cytokine deprivation, DNA damage (IR or etoposide), glucocorticoids (dexamethasone), or endoplasmic reticulum (ER) stress (tunicamycin) (Fig. 3 and fig. S9). *Puma*-deficient cells were less resistant to apoptosis than TKO cells (fig. S10). Indeed, deficiency of *Bid*, *Bim*, and *Puma* appears to provide more protection against intrinsic apoptotic death than deficiency of *Apaf-1* or *Capase-9* (28). By analogy to *Bax*^{-/-}*Bak*^{-/-} T cells (21), *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} T cells were still sensitive to Fas-induced apoptosis because BCL-2 family does not regulate extrinsic apoptotic death in T cells (Fig. 3E). The proapoptotic activity of BID is activated upon its cleavage by proteases such as caspase-8 or 2 (1, 4, 5). Cleavage of BID was detected in thymocytes in response to DNA damage or ER stress (fig. S11).

To investigate whether activation of BAX and BAK was blocked in the absence of *Bid*, *Bim*, and *Puma*, gel filtration was performed to assess homo-oligomerization of BAX or BAK. In both wild-type (WT) and *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO cells, BAX was eluted as a 20-kD monomer from untreated cells (Fig. 4A). After IR or treatment of cells with tunicamycin, BAX formed higher-ordered oligomers in WT but not TKO cells (Fig. 4A). Similarly, higher-ordered oligomers of BAK were only detected in WT cells in response to genotoxic or ER stress (Fig. 4B). Consistent with the lack of homo-oligomerization of BAX or BAK detected in TKO cells, cytochrome *c* translocation and caspase activation were not observed in these cells when exposed to diverse intrinsic apoptotic signals (Fig. 4, C and D, and figs. S12 and S13). Although the majority of WT cells lost cytochrome *c* immunostaining within 20 hours after IR, in TKO cells, cytochrome *c* remained in the mitochondria, exhibiting a punctate staining pattern (Fig. 4C). Caspase activation determined by the cleavage of poly (ADP-ribose) polymerase (PARP) and caspase-3 was not observed in TKO cells treated with IR, dexamethasone, or tunicamycin (Fig. 4D). Notably, tBID, BIM, and PUMA were potent death agonists in both WT and *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO cells (Fig. 4E and fig. S14).

The lack of strong and stable interaction between BH3s and BAX/BAK was thought to support the indirect activation model for BAX and BAK. However, dynamic interactions occur between BAX and activator BH3s, including tBID, BIM, and PUMA (18), which helps explain the previous difficulty in detecting these interactions. Here, we demonstrate an essential role of BID, BIM, and PUMA in activating BAX and BAK and for some apoptotic events during development. Our genetic study indicates that BAD is unable to induce apoptosis in the absence of BID, BIM, and PUMA, which is further supported by the observation that the BAD mimetic, ABT-737 (29), failed to kill *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO cells (fig. S15). These data suggest that the profound block of apoptosis conferred by the triple deficiency of *Bid*, *Bim*, and *Puma* is not simply caused by altering the ratio between anti-apoptotic and proapoptotic BCL-2 proteins. BH3s not only induce BAX- and BAK-dependent release of cytochrome *c* to activate caspases but also initiate caspase-independent mitochondrial dysfunction (9). Accordingly, persistent interdigital webs and accumulation of hematopoietic cells were observed in *Bax*^{-/-}*Bak*^{-/-} DKO (21) or *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO mice, but not in *Apaf-1*-deficient mice (28, 30). Overall, our study reveals an essential axis of activator BH3s and BAX and BAK in activating the mitochondrial death program, which offers common ground for therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

1. Gross A, McDonnell JM, Korsmeyer SJ. *Genes Dev.* 1999; 13:1899. [PubMed: 10444588]
2. Wang X. *Genes Dev.* 2001; 15:2922. [PubMed: 11711427]
3. Newmeyer DD, Ferguson-Miller S. *Cell.* Feb 21.2003 112:481. [PubMed: 12600312]
4. Reed JC. *Cell Death Differ.* Aug.2006 13:1378. [PubMed: 16729025]
5. Youle RJ, Strasser A. *Nat Rev Mol Cell Biol.* Jan.2008 9:47. [PubMed: 18097445]
6. Desagher S, et al. *J Cell Biol.* 1999; 144:891. [PubMed: 10085289]
7. Wei MC, et al. *Genes Dev.* Aug 15.2000 14:2060. [PubMed: 10950869]
8. Wei MC, et al. *Science.* 2001; 292:727. [PubMed: 11326099]
9. Cheng EH, et al. *Mol Cell.* Sep.2001 8:705. [PubMed: 11583631]
10. Letai A, et al. *Cancer Cell.* Sep.2002 2:183. [PubMed: 12242151]
11. Kuwana T, et al. *Mol Cell.* Feb 18.2005 17:525. [PubMed: 15721256]
12. Kim H, et al. *Nat Cell Biol.* Dec.2006 8:1348. [PubMed: 17115033]
13. Galonek HL, Hardwick JM. *Nat Cell Biol.* Dec.2006 8:1317. [PubMed: 17139279]
14. Kuwana T, et al. *Cell.* Nov 1.2002 111:331. [PubMed: 12419244]
15. Lovell JF, et al. *Cell.* Dec 12.2008 135:1074. [PubMed: 19062087]
16. Gavathiotis E, et al. *Nature.* Oct 23.2008 455:1076. [PubMed: 18948948]
17. Willis SN, et al. *Science.* Feb 9.2007 315:856. [PubMed: 17289999]
18. Kim H, et al. *Mol Cell.* Nov 13.2009 36:487. [PubMed: 19917256]
19. Certo M, et al. *Cancer Cell.* May.2006 9:351. [PubMed: 16697956]
20. Fu NY, Sukumaran SK, Kerk SY, Yu VC. *Mol Cell.* Jan 16.2009 33:15. [PubMed: 19150424]
21. Lindsten T, et al. *Mol. Cell.* 2000; 6:1389. [PubMed: 11163212]
22. Tronche F, et al. *Nat Genet.* Sep.1999 23:99. [PubMed: 10471508]
23. Miller TM, et al. *J Cell Biol.* Oct 6.1997 139:205. [PubMed: 9314540]
24. Putcha GV, et al. *J Cell Biol.* Apr 29.2002 157:441. [PubMed: 11980919]
25. Besirli CG, Wagner EF, Johnson EM Jr. *J Cell Biol.* Aug 1.2005 170:401. [PubMed: 16061693]
26. Harris CA, Johnson EM Jr. *J Biol Chem.* Oct 12.2001 276:37754. [PubMed: 11495903]
27. Chipuk JE, et al. *Proc Natl Acad Sci U S A.* Dec 23.2008 105:20327. [PubMed: 19074266]
28. Marsden VS, et al. *Nature.* Oct 10.2002 419:634. [PubMed: 12374983]
29. Oltersdorf T, et al. *Nature.* Jun 2.2005 435:677. [PubMed: 15902208]
30. Yoshida H, et al. *Cell.* 1998; 94:739. [PubMed: 9753321]
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Fig. 1.

Triple deficiency of *Bid*, *Bim*, and *Puma* phenocopies double deficiency of *Bax* and *Bak*. (A) *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO mice display persistence of interdigital webs. Ventral views of paws from WT, *Bid*^{-/-}*Bim*^{-/-}, and *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} mice. (B) *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO mice fail to develop external vaginal introitus. Photographs of vaginal openings from WT, *Bid*^{-/-}*Bim*^{-/-}, and *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} mice. Arrows point to external vaginal region. (C to G) Immunohistochemistry for cleaved caspase-3 from cerebella of postnatal day 5 (P5) mice of the indicated genotypes that were irradiated with 14 Gy γ -irradiation. (C) WT, 18 hours after IR. (D) *Bax*^{f/f}*Bak*^{-/-}*Nestin-Cre*⁺, 30 hours after IR. (E) *Puma*^{-/-}, 30 hours after IR. (F) *Bim*^{-/-}*Puma*^{-/-}, 30 hours after IR. (G) *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-}, 30 hours after IR. Arrows denote the external granular layer of cerebellum. Data shown are representative images of two to three independent experiments. (H) Analyses of a whole sagittal section of cerebellum at the same level from experiments shown in (C to G) summarize the numbers of neurons at the external granular layer that were stained positive for cleaved caspase-3. Data shown are the average of two independent experiments (n = 2).

Fig. 2. *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO cerebellar granule neurons are completely resistant to potassium-deprivation induced apoptosis. Cerebellar granule neurons from WT, *Bim*^{-/-}, *Puma*^{-/-}, *Bim*^{-/-}*Puma*^{-/-}, or *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} mice were cultured in high-K⁺ (K25 + S) for 7 days and then transferred to low-K⁺ medium (K5 + S) to induce apoptosis. Viability was determined at the indicated times using propidium iodide (PI) staining. Data are the mean percentage of PI-positive neurons ± SD from three independent experiments. **, *P* < 0.01; ***, *P* < 0.001.

Fig. 3.

Bid^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO T cells are resistant to diverse apoptotic stimuli. (A to E) CD4⁺ T cells purified from the spleens of WT (n = 3), *Bid*^{-/-}*Bim*^{-/-} (n = 3), or *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} (n = 3) mice at 8 to 10 weeks of age were cultured under the following conditions: in the absence of cytokine (A), after exposure to 2.5 Gy γ -irradiation (B), in the presence of etoposide (C), in the presence of dexamethasone (D), or in the presence of agonistic antibody to Fas (E). Cell death was quantified by annexin-V staining at the indicated times. (F to I) Thymocytes from WT (n = 3), *Bid*^{-/-}*Bim*^{-/-} (n = 3), or *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} (n = 3) mice at 6 to 8 weeks of age were cultured under the following conditions: in the absence of cytokine (F), after exposure to 2.5 Gy γ -irradiation (G), in the presence of etoposide (H), or in the presence of tunicamycin (I). Cell death was quantified by annexin-V staining at the indicated times. Data are the mean percentage of annexin-V-positive cells $\pm$ SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 4.

BID, BIM, and PUMA are required to activate BAX- and BAK-dependent mitochondrial apoptosis. (A and B) Triple deficiency of *Bid*, *Bim*, and *Puma* prevents activation of BAX and BAK. Thymocytes from WT or *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} mice were untreated, irradiated with 5 Gy γ -irradiation (IR), or treated with tunicamycin (TC). Protein lysates were harvested at 7 hours after IR or 20 hours after TC treatment and subjected to Superdex 200 (HR10/30) gel-filtration chromatography. Fractions were analyzed by Western blot using antibodies to BAX (A) or BAK (B). (C) Triple deficiency of *Bid*, *Bim*, and *Puma* prevents the translocation of cytochrome c. Fluorescence microscopy of WT or *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} thymocytes 20 hours after exposure to 2.5 Gy γ -irradiation. Red represents cytochrome c

immunostaining, and blue is Hoechst staining. White asterisks indicate apoptotic cells that have lost cytochrome c. **(D)** Triple deficiency of *Bid*, *Bim*, and *Puma* prevents the activation of caspases. Thymocytes from WT or *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} mice were untreated, treated with TC or dexamethasone (Dex), or irradiated with 5 Gy γ -irradiation (IR). After 12 hours, protein lysates were harvested and assessed by Western blot using antibodies specific for PARP, cleaved PARP, cleaved caspase-3, or actin. Asterisk denotes a cross-reactive protein. **(E)** Primary mouse embryonic fibroblasts isolated from WT or *Bid*^{-/-}*Bim*^{-/-}*Puma*^{-/-} TKO mice were infected with retroviruses expressing the indicated genes. Cell death was quantified by annexin-V staining at 24 hours. Data are the mean percentage of annexin-V-positive cells $\pm$ SD from three independent experiments. *, $P < 0.05$.