Blk, a BH3-containing Mouse Protein That Interacts with Bcl-2 and Bcl-xL, Is a Potent Death Agonist*

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We identified and cloned a novel murine member of the pro-apoptotic Bcl-2 family. This protein, designated Blk, is structurally and functionally related to human Bik and localized to the mitochondrial membrane. Blk contains a conserved BH3 domain and can interact with the anti-apoptotic proteins Bcl-2 and Bcl-xL. Ectopic expression of Blk in mammalian cells induces apoptosis, which can be inhibited by mutations in the BH3 domain and by overexpression of Bcl-2 or Bcl-xL but not by CrmA. The apoptotic activity of Blk is also inhibited by a dominant negative caspase-9, suggesting that Blk induces apoptosis through activation of the cytochrome c-Apaf-1-caspase-9 pathway.

Bcl-2 is the first recognized component of programmed cell death or apoptosis (1, 2), an evolutionary conserved process essential for normal development of multicellular organisms and in maintaining tissue homeostasis. Various aspects of involvement of Bcl-2 in this process have been well documented at the molecular and physiological levels (for review see Ref. 3). Bcl-2 is a multifunctional 239-amino acid protein that has a hydrophobic C-terminal membrane anchor that is preceded by three well recognized domains designated BH1, BH2, and BH3 (Bcl-2 homology domains 1, 2, and 3) that are necessary for its function (3). Bcl-2 and its homolog Bcl-xL are death antagonists that associate mainly with the outer mitochondrial membrane, the endoplasmic reticulum, and nuclear envelope and have well documented ion channel activity (3). Recent evidence suggests that they prevent apoptosis by regulating the electrical and osmotic homeostasis of the mitochondria, a process that is required to prevent mitochondrial swelling, outer membrane rupture, and cytochrome c release (4). Cytochrome c release from the mitochondria is believed to trigger activation of the death caspase cascade through formation of the Apaf-1-caspase-9-cytochrome c complex (5, 6).

Intriguingly, among the members of the Bcl-2 family of proteins discovered in recent years there are death agonists (e.g. Bax, Bad, Bik, Bak, Bid, and Hrk) (7–9). Except for Bad whose BH3 domain is within the putative BH1 domain, all of these proteins contain an independent BH3 domain (10). It appears that only the BH3 domain is required for their proapoptotic activity (8–15). This domain interacts with a hydrophobic cleft formed by the BH1, BH2, and BH3 domains of the anti-apoptotic Bcl-xL and Bcl-2 as evident from mutational and structural studies (15). Interestingly, Bik, Bid, and Hrk, which contain only a BH3 domain, seem to be more potent death effectors than those proteins with all the three domains of Bcl-2 (Bax and Bak) (Ref. 8 and this report). However, the molecular determinants of the “BH3-only” proteins as opposed to those of “all BH domains” proteins that trigger the key components of the death pathway(s) leading to activation of the terminal caspases remain to be understood. It is also not clear whether binding of the BH3 domain of the death agonist inactivates Bcl-2/Bcl-xL by simple stoichiometric binding or whether it initiates a new activity that triggers apoptosis.

Here we report the identification and characterization of a novel mouse proapoptotic protein designated Blk. The protein, which has 43% homology with the human Bik/Nbk (12, 16), contains a well conserved BH3 domain, binds human Bcl-2 and Bcl-xL, and appears to be a potent death agonist like Bik (BH3-only protein) and Bax (all BH domains protein) as tested in human cell lines. The death effector BH3 domain was confirmed by mutational studies. Furthermore, the involvement of caspases in Bcl-2-induced apoptosis was tested by using a dominant negative inhibitor approach, which provides evidence that caspase-9 (Mch6/ICE-LAP6) (17, 18) is the most upstream caspase in BH3 domain-induced apoptosis.

MATERIALS AND METHODS

cDNA Cloning—The full-length Blk cDNA was cloned from a mouse 14.5-day-old embryonic liver cDNA library (random primed) by PCR. This was done using primers corresponding to the 3′ region of mouse GenBank™ expressed sequence tag clone 694727 as antisense primers and vector specific primers (Agt11 forward or reverse) as sense primers.

Mammalian Expression Vectors—FLAG and T7 epitope tagging was done by cloning the PCR-generated cDNAs of the respective genes into pFLAG CMV-2 and pcDNA-3-T7 vectors, respectively. To generate N-terminal GFP-tagged Blk, its cDNA was inserted in-frame into the expression vector pEGFP-C1 (CLONTECH). For apoptosis assays, we used the mammalian double expression vector pRSC-LacZ (19), which allows the expression of LacZ under the Rous sarcoma virus promoter, and the test cDNA (Blk, Bik, Bax, and Bik BH3 mutants) under the CMV promoter.

In Vitro and in Vivo Interaction Assays—GST fusion proteins were expressed in Escherichia coli and purified by affinity chromatography. 35S-Labeled proteins were expressed in vitro using a coupled transcription/translation system in rabbit reticulocyte lysate using a TNT kit (Promega) according to the manufacturer’s recommendations. In vitro protein-protein interaction was carried out according to Boyd et al. (12). Briefly, the labeled proteins were precleared by mixing with glutathione-Sepharose beads and then removed by centrifugation. The supernatants were incubated with GST-Bcl-2, GST-Bcl-xL, or GST immobilized on glutathione-Sepharose beads for 2 h at 4 °C. After extensive washing, the interacting proteins were recovered by boiling the beads in SDS sample buffer and analyzed on 12% SDS-PAGE. For in vivo inter-
boxed residues are boxed. The 12-amino acid BH3 domain is boxed and shaded. Asterisks indicate residues that are absolutely conserved in the BH3 domains of all mammalian Bcl-2 family members. B, Northern blot analysis of the expression of murine Blk in adult mouse tissues. The Blk mRNA was detected with a Blk-specific radiolabeled riboprobe. The size of the Blk mRNA in kilobases (0.85 kilobases) is indicated to the right.

action of T7-tagged Bcl-2 and Bcl-xL with the death agonist (FLAG-tagged blk, Bik, and Bax), the corresponding proteins were co-expressed in human embryonic kidney 293 cells, and the interacting proteins were isolated by immunoprecipitation with anti-FLAG monoclonal antibody, followed by immunoblot analysis with an horseradish peroxidase-conjugated T7 antibody as described before (20).

Mutagenesis—The conserved amino acids in the BH3 domain of Blk were mutated by site-directed mutagenesis using overlapping PCR as described before (17). The generated PCR products were cloned in pFLAG CMV-2 and pBSC-double vector, and the mutations were confirmed by sequencing.

RESULTS AND DISCUSSION

Identification and Cloning of a Murine Death Agonist—To identify additional members of the BH3-only proteins that are involved in apoptosis, we searched the GenBank® expressed sequence tag data base for sequences that encode conserved BH3 domain. A mouse partial cDNA clone was identified. Based on its 3′ sequence, PCR primers were generated and used to clone a full-length cDNA that encodes a new Bik-related protein of 150 amino acids (Fig. 1A). This protein shares an overall ~43% identity with human Bik and a fully conserved BH3 domain of 12 amino acids (Fig. 1A) that is also present in other Bcl-2 family members (12, 16). However, the two proteins are less conserved in the regions N-terminal and C-terminal to the BH3 domain. There are also five deletions in the new protein that make it 10 amino acids shorter than Bik. Based on these criteria and its apoptotic activity (see below), the new protein was designated Bik-like killer (Blk).

Tissue Distribution of Blk—To determine the distribution of Blk, various mouse tissue mRNA samples were subjected to Northern blot analysis. As shown in Fig. 1B, the Blk riboprobe detected a ~0.85-kilobase transcript in the testes, kidney, liver, lung, and heart but not in skeletal muscle, spleen, and brain. The highest expression was seen in the liver and kidney. This restricted expression is different from that observed with human Bik, which is widely expressed in both adult tissues and established cell lines (12). Interestingly, the mRNA expression of murine Bid, another BH3-only apoptotic protein, has been shown to be relatively abundant in brain and spleen tissues (8).

Blk Is Localized to the Mitochondrial Membrane—A charac-

FIG. 1. Amino acid sequence and tissue distribution of Blk. A, alignment of murine Blk and human Bik protein sequences. Identical residues are boxed. The 12-amino acid BH3 domain is boxed and shaded. Asterisks indicate residues that are absolutely conserved in the BH3 domains of all mammalian Bcl-2 family members. B, Northern blot analysis of the expression of murine Blk in adult mouse tissues. The Blk mRNA was detected with a Blk-specific radiolabeled riboprobe. The size of the Blk mRNA in kilobases (0.85 kilobases) is indicated to the right.

FIG. 2. Subcellular localization of Blk. MCF-7 cells were transfected with a GFP-Blk expression vector (D–F) or a GFP control vector (A–C) and incubated for 16 h at 37 °C. The transfected cells were fixed and then stained with the mitochondrial specific stain MitoTracker® Red CMXRos (Molecular Probes, Inc.). Cells were visualized by confocal laser scanning microscopy. A–C, same field of GFP control cells visualized by green fluorescence (A), MitoTracker® Red CMXRos (B) or both (C), D–F, same field of GFP-Blk cells visualized by green fluorescence (D), MitoTracker® Red CMXRos (E), or both (F).

Blk Is a Potent Death Effector, and Its Apoptotic Activity Is Inhibited in the Presence of Co-expressed Bcl-2 or Bcl-xL—The apoptotic proteins of the Bcl-2 family (e.g. Bax) are believed to initiate the apoptotic process stoichiometrically, where the death or survival of the cell depends upon the ratio of the apoptotic to the anti-apoptotic proteins in the cell (21). To examine the apoptotic activity of Blk compared with Bik and Bax, increasing amounts of constructs expressing these proteins were transfected into MCF-7 cells, and the number of apoptotic cells were scored. Consistent with previous observations, the three proteins induced apoptosis in a concentration-dependent manner, where the apoptotic effect was dependent on the amount of DNA transfected (Fig. 3A). Interestingly, in all our experiments, murine Blk showed higher potency than Bik and Bax. Similar results were observed in the human embryonic kidney 293 cells (data not shown).

Consistent with their ability to antagonize the death promoting activity of the proapoptotic Bcl-2 family members, transfected Bcl-2 and Bcl-xL were able to suppress the proapoptotic activity of Blk, Bik, and Bax (Fig. 3B). Bcl-xL was a better
inhibitor of apoptosis than Bcl-2 in all these cases. The extent of Bcl-2/Bcl-xL-mediated inhibition of apoptosis induced by the three death agonists followed the order Blk < Bik < Bax. This is in correlation with the ability of Blk to induce more apoptosis than Bik and Bax (Fig. 3A). In addition, the inhibition of apoptosis depends on the ratio of Bcl-2 or Bcl-xL to Blk, because increasing this ratio results in more inhibition of apoptosis (Fig. 3C). These data support earlier observations that the ratio of the pro- to anti-apoptotic Bcl-2 proteins determines whether cells die or survive.

Blk Binds Bcl-2 and Bcl-xL—All the proapoptotic Bcl-2 family members heterodimerize with Bcl-2 and Bcl-xL. To determine whether Blk can also bind to Bcl-2 or Bcl-xL, in vivo and in vitro binding studies were performed. 35S-Labeled Blk, Bik, or Bax were precipitated with GST, GST-Bcl-2, or GST-Bcl-xL fusion proteins immobilized on glutathione-Sepharose beads (Fig. 4A). Like Bik and Bax, Blk specifically associated with GST-Bcl-2 and GST-Bcl-xL but not with the GST control. This suggests that Blk interacts directly with Bcl-2 and Bcl-xL. Similarly, Blk, Bik, and Bax bound to Bcl-2 and Bcl-xL when these proteins were co-expressed in vivo (Fig. 4B).

**The BH3 Domain of Blk Is Critical for Its Proapoptotic Activity and Interaction with Bcl-2 and Bcl-xL**—In view of the importance of the BH3 domain in the death agonist activity of the proapoptotic Bcl-2 family members (8–15), we introduced mutations in the BH3 domain of Blk to identify critical residues that are essential for its function. The Leu55 and Gly59-Asp60 of Blk are conserved in the BH3 domains of the Bcl-2 family members (Fig. 1A). Mutations in these three residues have been shown to affect both protein-protein interaction and apoptotic efficiency (8–15).

To determine the positional importance of Gly59-Asp60 on the function of Blk, we generated two Blk mutants in which either Gly59 (Blk-G59) or Gly59 and Asp60 (Blk-G59-D60) were deleted. As shown in Fig. 4 (C and D), Blk–G59 showed drastic reduction in both binding to Bcl-2 and Bcl-xL and death agonist activity. Deletion of Gly59-Asp60 abrogated both activities which is consistent with that observed in the all BH domains protein Bak (11). This result demonstrates the importance of these residues in keeping the overall geometry of the BH3 domain, and further confirms the critical role of the BH3 domain in the death agonist activity of Blk.

**Active Site Mutation of Caspase-9 Blocks Blk, Bik, and Bax-Induced Apoptosis**—Cytochrome c release from the mitochondria and its binding to Apaf-1 triggers a dATP/ATP-dependent activation of caspase-9, the most upstream caspase in the cytochrome c-dependent apoptotic pathway (5). This pathway can
polyclonal antibody against human caspase-3 p20 (upper panel) or a monoclonal antibody to the FLAG epitope (lower panel).

be blocked by an active site dominant negative caspase-9 mutant (5). Bcl-2 and Bcl-xL can also block this pathway possibly by preventing cytochrome c release from the mitochondria (6). To determine whether the cytochrome c-Apaf-1-caspase-9 pathway is involved in Blk-induced apoptosis, MCF-7 cells were co-transfected with Blk, Bik, or Bax and an inactive caspase-9 C287A mutant. Overexpression of Blk, Bik, and Bax induced apoptosis in nearly 97, 78, and 70% of the cells, respectively (Fig. 5A). The active site mutant of caspase-9 reduced the percentage of cells undergoing apoptosis to nearly 52, 20, and 10%, respectively. When caspase-9 mutant was substituted for wt caspase-9 (Fig. 5B). This processing was almost completely blocked when a 4-fold excess of mutant caspase-9 was co-expressed with Blk or Bik (Fig. 5B). Processing of caspase-7 was also inhibited in these cells (data not shown) by the caspase-9 mutant, suggesting that caspase-3 and -7 are downstream of caspase-9 in Blk-induced apoptosis. Taken together, caspase-9 appears to be the most upstream caspase in the apoptotic pathway triggered by the proapoptotic Bcl-2 family members.

In conclusion, Blk was identified as a novel member of the Bcl-2 family of death regulatory proteins. Like Bik, Bid, and Btk, Blk shares sequence homology with other family members in the BH3 domain but lacks the conserved BH1, BH2, and BH4 domains. The BH3 domain is essential for the death agonist activity of Blk and its ability to interact with Bcl-2 and Bcl-xL, because deletions of conserved residues in this domain abolished both activities. This supports a model suggesting that direct interactions between the BH3 domain of Blk and Bcl-2 or Bcl-xL is required to suppress the protective activity of these proteins and induce apoptosis. Our data also suggest that Blk, Bik, and Bax induce apoptosis by triggering the formation of the Apaf-1-caspase-9 complex and activation of caspase-9, downstream of Bcl-2 and Bcl-xL. The precise mechanism by which they trigger this process is not yet known. However, because cytochrome c is required for formation of the Apaf-1-caspase-9 complex (5), we hypothesize that they may trigger cytochrome c release from the mitochondria by inhibiting the channel forming activity of Bcl-2 or Bcl-xL (4), which may lead to disruption of the outer mitochondrial membrane.

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REFERENCES

1. Tsujimoto, Y., Jaffe, E., Cosman, J., and Croce, C. M. (1986) Science 228, 1440–1443
2. Nunez, G., London, L., Hockenberg, D., Alexander, M., McKearn, J. P., and Korsmeyer, S. J. (1990) J. Immunol. 144, 3602–3610
3. Reed, J. C. (1997) Nature 387, 773
4. Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. (1997) Cell 91, 627–637
5. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
6. Reed, J. C. (1997) Cell 91, 559–562
7. Hawkins, C. J., and Vaux, D. L. (1997) Semin. Immunol. 9, 25–33
8. Wang, K., Yin, X.-M., Chao, D. T., Milliman, C. L., and Korsmeyer, S. J. (1997) Genes Dev. 10, 2859–2869
9. Inohara, N., Ding, L., Chen, S., and Nunez, G. (1997) EMBO J. 16, 1686–1694
10. Zha, J., Harada, H., Osipov, K., Joekel, J., Walksman, G., and Korsmeyer, S. J. (1997) J. Biol. Chem. 272, 24101–24104
11. Chittenden, T., Flemington, C., Houston, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G., and Lutz, R. J. (1995) EMBO J. 14, 5589–5596
12. Boyd, J. M., Gallo, G. J., Elangovan, G., Houghton, A. B., Malstrom, S., Avery, B. J., Ebb, R. G., Subramanian, T., Chittenden, T., Lutz, R. J., and Chinnadurai, G. (1995) Oncogene 11, 1921–1928
13. Zhu, H., Aime-Sepme, C., Saijo, T., and Reed, J. C. (1997) J. Biol. Chem. 272, 7440–7444
14. Hunter, J. J., and Parslow, T. G. (1996) J. Biol. Chem. 271, 8521–8524
15. Sattler, M., Liang, H., Nettseth, D., Meadows, R. P., Harlan, J. E., Ellis, Shaker, M. Y., Yu, H. S., Shaker, S. B., Chang, B. S., Mint, A. J., Thompson, C. B., and Fesik, S. N. (1997) Science 275, 1129–1132
16. Han, J., Sabbatini, P., and White, E. (1996) Mol. Cell. Biol. 16, 5857–5864
17. Srinivasula, S. M., Ahmad, M., Fu, J., Kitajewski, J., Korsmeyer, S. J. (1997) J. Biol. Chem. 272, 1921–1928
18. Han, J., Sabbatini, P., and White, E. (1996) Mol. Cell. Biol. 16, 5857–5864
19. Srinivasula, S. M., Ahmad, M., Ottilie, S., Bullrich, F., Banker, S., Wang, Y., Fernandes-Alnemri, T., Croce, C. M., Litwack, G., Tomaselli, K. J., Armstrong, R. C., Wang, L., Trapani, J. A., Tomaselli, K. J., Armstrong, R. C., Wang, L., Trapani, J. A., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996) J. Biol. Chem. 271, 27099–27106
20. Duan, H., Orth, K., Chinmayan, A., Purrier, G., Froedich, C. J., He, W., and Dixit, V. M. (1997) J. Biol. Chem. 272, 16729–16734
21. MacFarlane, M., Ahmad, M., Srinivasula, S. M., Fernandes-Alnemri, T., Cohen, G. M., and Alnemri, E. S. (1997) J. Biol. Chem. 272, 29417–29420
22. Srinivasula, S. M., Ahmad, M., Ottilie, S., Bullrich, F., Banker, S., Wang, Y., Fernandes-Alnemri, T., Croce, C. M., Litwack, G., Tomaselli, K. J., Armstrong, R. C., and Alnemri, E. S. (1997) J. Biol. Chem. 272, 18542–18545
23. Oltvai, Z. N., and Korsmeyer, S. J. (1996) Cell 89, 189–192
24. Orth, K., and Dixit, V. M. (1997) J. Biol. Chem. 272, 8841–8844
25. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13706–13711
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