Bik and Bak Induce Apoptosis Downstream of CrmA but Upstream of Inhibitor of Apoptosis*

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Recent studies have identified a number of cell death pathway components. In this study, we describe the role that two such components, Bik and Bak, play in initiating the apoptotic program. These Bcl-2 family members engage the death pathway downstream of the block imposed by the serpin CrmA, but upstream of the block initiated by cellular inhibitors of apoptosis, which are a family of molecules characterized by a conserved baculovirus inhibitor of apoptosis repeat motif. Distal death pathway components activated by Bik and Bak are similar to those activated by the CD-95 (Fas/Apo1) and tumor necrosis factor death receptors.

Apoptosis or programmed cell death plays a critical role in the survival of multicellular organisms. Unchecked, it can contribute to a number of degenerative conditions such as Alzheimer’s disease (1, 2). Conversely, excessive inhibition of cell death can lead to the accumulation of abnormal cells that may become transformed or contribute to autoimmune disease (3). Therefore, preserving the balance between pro- and anti-apoptotic influences is essential for maintaining tissue homeostasis (4).

Genetic studies in Caenorhabditis elegans have contributed much to the understanding of key components of the cell death machinery by the identification of an inhibitor of apoptosis, ced-9, and two inducers of apoptosis, ced-3 and ced-4 (5). The ced-9 gene is homologous to the human bcl-2 gene, which is overexpressed in follicular lymphomas and contributes to a heightened state of resistance to cell death induced by a variety of agents including glucocorticoids and irradiation (6–9). Although the mammalian homologue of ced-4 has yet to be identified, CED-3 is homologous to a family of interleukin-1-converting enzyme-like proteases (ICE) recently renamed caspases (10, 11). Caspases are cysteine proteases that cleave substrates following aspartate residues and when overexpressed can induce apoptosis (12, 13). In caspases, aspartate are converted from an inactive zymogen form to a cleaved active dimeric species upon receipt of a death signal (14, 15).

Although the components identified in C. elegans have revealed some of the essential mammalian counterparts involved in apoptosis, additional complexity exists in mammalian systems. A number of receptors involved in immune function that contain death domains, including CD-95, tumor necrosis factor receptor-1 (TNFR1 or p55), and DR3/WSL-1, can engage the death pathway by using adapter molecules such as FADD/MORT1 to directly recruit a proximal caspase (caspase-8/FLICE/MACH) (16–25). Additionally, while there is only one ced-9 gene in C. elegans, there are a number of Bcl-2 homologues in mammals that can either suppress (Bcl-x) (26) or activate (Bax, Bik, Bak) (27–31) the apoptotic program. Superimposed on this are viral inhibitors of cell death that attenuate the pathway allowing for increased replication of viral progeny in infected host cells. The pox virus-encoded serpin, CrmA, preferentially inhibits proximal components of the ICE/CED-3 protease cascade, including caspase-8 (32, 33). In contrast, the baculoviral encoded gene product p35 interrupts the death pathway by inhibiting a broad spectrum of caspases (34, 35). Additional baculoviral gene products that inhibit apoptosis are the IAP molecules (Cp-IAP and Op-IAP) that are characterized by protein repeat (BIR) domains (36). While the exact mode of action of these inhibitors is unclear, homologues in Drosophila and humans have been identified (37–41). Remarkably, deficiency of one of the human IAP genes (NIAP) may contribute to the excessive neuronal apoptosis that characterizes spinal muscular atrophy (42, 43).

To gain a better understanding of how these different modulators and effectors of apoptosis might function, we investigated the activities of two activators of apoptosis, Bik and Bak, with respect to various inhibitors of the cell death program. Herein, we show that the activation of apoptosis by Bik and Bak occurs downstream of the CrmA block. However, a broad spectrum peptide inhibitor of the caspase family, z-VAD-fmk, attenuated cell death induced by Bik or Bak, implying a down-stream effector role for caspases. Confirming this is the finding that caspase-7, a distal caspase, is processed to its active form upon induction of cell death by Bik or Bak. Surprisingly, the mammalian counterparts to the baculoviral IAPs (cIAP1 and cIAP2) inhibited Bik- and Bak-induced apoptosis. This allows for the derivation of a model that tentatively positions the aforementioned components in the apoptotic program.

MATERIALS AND METHODS

Cell Lines—MCF7, a human breast carcinoma cell line transfected with either vector (Neo-vector) or a CrmA expression construct (Neo-CrmA) (33) was maintained in RPMI containing 10% heat-inactivated fetal bovine serum. The HA-Bik and HA-Bak constructs were generously provided by G. Chinnadurai. The myc-IAP1 and myc-IAP2 constructs were generously provided by D. V. Goeddel. The p55 TNF receptor construct was described previously in Ref. 19.

Death Assays—These were performed essentially as described previously (44). Briefly, parental, Neo-vector or Neo-CrmA cells were plated on a six-well tissue culture dish (2 × 10^5 cells/well) and transiently transfected with 100 ng of the reporter plasmid pCMV-β-galactosidase plus 0.2 μg of the test plasmid. Inhibition assays included 0.8 μg of the inhibitor test plasmid. Five hours following transfection, 1 ml of complete medium (in the presence or absence of 40 μM z-VAD-fmk) was added. Nonapoptotic or apoptotic cells were detected by staining with 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside as described previ
RESULTS AND DISCUSSION

Bik and Bak Activate Programmed Cell Death Downstream of the CrmA Block—CrmA is a virally encoded serpin that inhibits both CD-95 and TNF-induced cell death (33). It apparently functions at the apex of these receptor-initiated cell death pathways based on its ability to block the activation of a number of downstream caspases, including caspase-3 (YAMA, CPP32, apopain), caspase-6 (Mch2), and caspase-7 (ICE-LAP3, Mch3, CMH-1) (20, 24, 33, 47, 48). Therefore, the protease(s) that CrmA inhibits must be upstream of these distal caspases. Additionally, staurosporine can by-pass the proximal CrmA block and induce activation of these same distal caspases (47, 48). Finally, CrmA is a relatively weak inhibitor of the distal caspases (caspase-3, caspase-6, and caspase-7), whereas it is a potent inhibitor of caspase-8, which functions at the apex of the cascade (32, 49). Using the previously characterized MCF7 vector or CrmA stable transfectants, we investigated whether the proapoptotic members of the Bcl-2 family, Bik and Bak, could by-pass the upstream CrmA block.

As observed previously, TNF induced apoptosis in the vector control cell line, whereas the CrmA cell line was protected (Fig. 1) (33). The vector control cell line transfected with Bik or Bak underwent apoptosis. However, the CrmA-expressing cell line also underwent apoptosis with similar kinetics. Identical results were observed when CrmA and Bik or Bak were transiently cotransfected into the parental MCF7 cell line, implying that the result was not attributable to clonal variation (data not shown). The gross morphology of the apoptotic cells induced to die by either overexpression of Bik or Bak or treatment with TNF was indistinguishable. Therefore, although Bik and Bak must initially activate a distinct pathway from that engaged by CD-95 or TNF (as revealed by CrmA sensitivity), downstream of CrmA the pathways are likely to be similar.

Bik and Bak Activate Caspases to Induce Cell Death—In vitro, caspases are potently inhibited by the cell permeable, broad spectrum peptide inhibitor z-VAD-fmk (50, 51). This peptide inhibitor abrogates both CD-95- and TNF-induced cell death (Fig. 2A) and attenuates a number of other forms of death, including glucocorticoid-induced death (8, 9). Likewise, Bik- and Bak-induced cell death was inhibited by z-VAD-fmk (Fig. 2A), consistent with death being mediated by caspase activation.

To confirm this, the activation of one of the distal caspases, caspase-7, was examined. The caspases are found as single polypeptide zymogens in live cells, whereas upon receipt of m
death signal (such as activation of CD-95 or TNF receptors or exposure to staurosporine) these proteases are processed into an active dimeric species (45, 47, 48). As shown in Fig. 2B, in mock-transfected cells (first lane) caspase 7 is present in itszymogen form, whereas in cells overexpressing the cysteopt55 TNF receptor (second lane) this caspase is converted to the active processed form as evidenced by the appearance of the large catalytic subunit (~20 kDa). Since Bik or Bak overexpression also resulted in the conversion of casapse-7 to its active form (Fig. 2B, third and fourth lanes, respectively), these proapoptotic Bcl-2 homologues must promote the activation of downstream caspases.

Finally, we analyzed the state of the endogenous apoptotic substrate, PARP (46). In nonapoptotic cells PARP is observed in the uncleaved form (116 kDa), whereas in apoptotic cells PARP is characteristically cleaved to an indicator apoptotic fragment (85 kDa) by a variety of active caspases (Fig. 2C, first and second lanes, respectively). As shown in Fig. 2C, cells transiently transfected with Bik (third lane) or Bak (fourth lane) contained this signature apoptotic PARP fragment that presumably was the result of cleavage by an activated endogenous caspase. This provides additional confirmation that Bik and Bak can by-pass the CrmA block and functionally activate downstream caspases.

**IAPs Inhibit Bik- and Bak-induced Cell Death**—By contrast to CrmA, the mammalian counterparts of the baculoviral IAP genes (cIAP1 and cIAP2) inhibited both Bik- and Bak-induced apoptosis. Cells transiently cotransfected with Bik or Bak and either cIAP1 or cIAP2 ( singly or together) did not undergo apoptosis (Fig. 3). Since no physical association between Bik or Bak and the cIAPs was observed (data not shown), it is likely that the effect involves an intermediary molecule(s). Regardless, Bik and Bak engage the death pathway upstream of the IAP block.

**Molecular Ordering of Bik and Bak in the Apoptotic Pathway**—Our results are consistent with a model whereby Bik and Bak trigger apoptosis by activation of caspases distal to the CrmA block but upstream of the IAPs. Our studies, however, do not address the question of whether Bik and Bak act by directly engaging the death machinery or by simply inhibiting an inhibitor of apoptosis such as Bcl-2. Finally, Bik and Bak were shown to promote the activation of distal caspases similar to those utilized by the CD-95 and TNF death pathways (Fig. 4).

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**Fig. 3. Bik- and Bak-induced cell death is inhibited by IAPs.** Apoptosis was triggered when MCF7 cells were transiently transfected with either Bik or Bak. By contrast, when MCF7 cells were transiently transfected with either Bik or Bak in combination with cIAP1 or cIAP2 (together or separately), cell death was attenuated.

**Fig. 4. Relative order of death pathway activators and inhibitors.** At the apex of the cascade are the receptor (CD-95 and TNF)-initiated death signals that are susceptible to CrmA inhibition. Downstream of this block, other Bcl-2-inhibitable death stimuli can enter the death pathway. Bik and Bak may inhibit Bcl-2 directly to release an unidentified cytotoxic factor. Alternatively, Bik and Bak may activate the pathway directly. Regardless IAPs function downstream of Bik and Bak. Activation of distal caspases results in cell death.

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**REFERENCES**

1. Vito, P., Wolozin, B., Ganjei, J. K., Iwasaki, K., Lacana, E., and D’Adamio, L. (1996) J. Biol. Chem. 271, 31025–31028
2. Vito, P., Lacana, E., and D’Adamio, L. (1996) Science 271, 521–525
3. Thompson, C. B. (1995) Science 267, 1456–1462
4. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
5. Horvitz, H. R., Shaham, S., and Hengartner, M. O. (1994) Cold Spring Harbor Symp. Quant. Biol. 59, 377–386
6. Alnemri, E. S., Fernandes, T. F., Haldar, S., Croce, C. M., and Litwack, G. (1992) Cancer Res. 52, 491–495
7. Hengartner, M. O., and Horvitz, H. R. (1994) Cell 76, 665–676
8. White, E. (1996) Genes Dev. 10, 1–15
9. Raff, M. C. (1992) Nature 356, 397–400
10. Cerdetti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., Ness, K. V., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A., Huebner, K., and Black, B. (1992) Science 255, 169–175
11. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molinex, S. M., Weidner, J. R., Aminis, J., Elliott, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J-P., Egger, L., A. Gaffney, E. P., Limjueco, G., Palyha, O. C., Raja, S. M., Rolando, A. M., Salley, J. P., Yam, T. I., Lee, T. D., Shively, J. E., Macross, M., Mambretti, R. A., Schmidt, J. A., and Tocci, M. J. (1992) Nature 365, 678–774
12. Miura, M., Zhu, H., Rotello, R., Hartwig, E. A., and Yuan, J. (1995) Cell 75, 653–660
13. Chinnaiyan, A. M., and Dixit, V. M. (1996) Curr. Biol. 6, 555–562
14. Kumar, S., and Harvey, N. L. (1995) FEBS Lett. 375, 169–173
15. Martin, S. J., and Green, D. R. (1995) Cell 72, 349–352
16. Ishii, N., and Nagata, S. (1996) J. Biol. Chem. 271, 30932–30937
17. Tartaglia, L. A., Rotte, M., Hu, Y. F., and Goeddel, D. V. (1993) Cell 73, 213–216
18. Nagata, S., and Golstein, P. (1995) Science 267, 1448–1456
19. Chinnaiyan, A. M., O’Rourke, K., Yu, G. L., Lyons, R. H., Molineaux, S. M., Weidner, J., Aminis, J., Elliott, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J-P., Egger, L. A., Gaffney, E. P., Limjueco, G., Palyha, O. C., Raja, S. M., Rolando, A. M., Salley, J. P., Yam, T. I., Lee, T. D., Shively, J. E., Macross, M., Mambretti, R. A., Schmidt, J. A., and Tocci, M. J. (1992) Nature 365, 678–774
20. Boldin, M. P., Varfolomeev, E. E., Panczer, Z., Mett, I. L., Canovis, J. H., and Wallach, D. (1995) J. Biol. Chem. 270, 7785–7789
21. Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Krammer, P. H., and Dixit, V. M. (1995) EMBO J. 14, 5579–5588
22. Boldin, M. P., Goncharo, T. M., Golstein, Y. V., and Wallach, D. (1996) Cell 85, 803–815
23. Munoz, M., Chinnaiyan, A. M., Kischkel, F. C., O’Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Brentjens, J. J., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., and Dixit, V. M. (1996) Cell 85, 817–827
24. Kitson, J., Raven, T., Jiang, Y.-P., Goeddel, D. V., Giles, K. M., Furn, K.-T., Grinham, C. J., Brown, R., and Farrow, S. N. (1996) Nature 384, 372–375
26. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993) Cell 74, 597–608
27. Oltavai, Z. N., Millian, C. L., and Korsmeyer, S. J. (1993) Cell 74, 609–619
28. Boyd, J. M., Gallo, G. J., Elangovan, B., Houghton, A. B., Malstrom, S., Avery, B. J., Ebb, R. G., Subramanian, T., Chittenden, T., Lutz, R. J., and Chinnadurai, G. (1995) Oncogene 11, 1921–1927
29. Kiefer, M. C., Brauer, M. J., Powers, V. C., Wu, J. J., Umansky, S. R., Tomei, L. D., and Barr, P. J. (1995) Nature 374, 736–739
30. Farrow, S. N., White, J. H., Martinou, I., Raven, T., Pun, K. T., Grinham, C. J., Martinou, J. C., and Brown, R. (1995) Nature 374, 731–733
31. Chittenden, T., Harrington, E. A., O'Connor, R., Flemington, C., Lutz, R. J., Evan, G. I., and Guild, B. C. (1995) Nature 374, 733–736
32. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) J. Biol. Chem. 272, 2952–2956
33. Tewari, M., and Dixit, V. M. (1995) J. Biol. Chem. 270, 3255–3260
34. Beidler, D. R., Tewari, M. T., Poirier, G., and Dixit, V. M. (1995) J. Biol. Chem. 270, 16526–16528
35. Bump, N. J., Hackett, M., Huginin, M., Sheshagiri, S., Brady, K., Chen, P., Perenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenburg, A. H., Miller, L. K., and Wong, W. W. (1995) Science 269, 1885–1888
36. Clem, R. J., and Miller, L. K. (1994) Mol. Cell. Biol. 14, 5212–5222
37. Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dagen, J. L., Gillilan, M. C., Shields, H., Hardwick, J. M., and Thompson, C. B. (1996) EMBO J. 15, 2685–2694
38. Hay, B. A., Wasserman, D. A., and Rubin, G. M. (1995) Cell 83, 1253–1262
39. Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J., Mackenzie, A., and Korneluk, R. G. (1996) Nature 379, 349–352
40. Rothe, M., Pan, M.-G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1995) Cell 83, 1243–1252
41. Uren, A. G., Pakusch, M., Hawkins, C. J., Puls, K. L., and Vaux, D. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4974–4978
42. Roy, N., Mahadevan, M. S., McLean, M., Shutler, G., Yaraghi, Z., Farahani, R., Baird, S., Beumer-Johnston, A., Lefebvre, C., Kung, X., Salik, M., Aubry, H., Tamai, K., Guan, X., Ioannou, P., Crawford, T. O., de Jong, P. J., Surh, L., Ikeda, J.-E, Korneluk, R. G., and Mackenzie, A. (1995) Cell 80, 167–178
43. Lefebvre, S., Burglen, L., Beausset, S., Clermont, O., Burlet, P., Violet, L., Benichou, B., Crasau, C., Millasseau, P., Zeviani, M., Le Paslier, D., Frezal, J., Cohen, D., Weissenbach, J., Munnich, A., and Melki, J. (1995) Cell 80, 155–165
44. Duan, H., Orth, K., Chinnaiyan, A. M., Poirier, G. G., Froelich, C. J., He, W. W., and Dixit, V. M. (1996) J. Biol. Chem. 271, 16720–16724
45. Duan, H., Chinnaiyan, A. M., Hudson, P. L., Wing, J. P., He, W., and Dixit, V. M. (1996) J. Biol. Chem. 271, 1621–1625
46. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) Cell 81, 801–809
47. Orth, K., Chinnaiyan, A. M., Garg, M., Froelich, C. J., and Dixit, V. M. (1996) J. Biol. Chem. 271, 16443–16446
48. Chinnaiyan, A. M., Orth, K., O'Rourke, K., Duan, H., Poirier, G. G., and Dixit, V. M. (1996) J. Biol. Chem. 271, 4573–4576
49. Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M., and Salvesen, G. (1997) J. Biol. Chem. 272, 750–757
50. Fearnhead, H. O., Dinsdale, D., and Cohen, G. M. (1995) FEBS Lett. 375, 283–288
51. Prong, G. J., Ramer, K., Amiri, P., and Williams, L. T. (1996) Science 271, 808–810
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