Bik is a potent pro-apoptotic protein, which complexes with various anti-apoptotic proteins such as Bcl-2, Bcl-x<sub>L</sub>, 19-kDa adenovirus E1B, and EBV-BHRF1. The mechanism by which Bik promotes cell death is not known. It shares a conserved domain, BH3, with other pro-apoptotic proteins, Bax, Bak, Bid, and Hrk, and certain anti-apoptosis proteins such as Bcl-2 and Bcl-x<sub>L</sub>. Mutations within the BH3 domain of Bik abrogate its ability to induce cell death and to complex with anti-apoptosis proteins. This result is consistent with the hypothesis that Bik may promote cell death by complexing with and antagonizing the activity of endogenous cellular anti-apoptosis proteins such as Bcl-2 and Bcl-x<sub>L</sub>. To elucidate the relationship between protein complex formation and induction of cell death, we have identified the minimal sequences of Bik, from a library of N-terminal and C-terminal deletion mutants, required for interaction with Bcl-2 and Bcl-x<sub>L</sub> and for inducing efficient cell death. Two-hybrid analysis in yeast and immunoprecipitation analysis of proteins expressed in mammalian cells indicate that a 52-amino acid region (amino acids 43–94) of Bik, encompassing the BH3 domain, is sufficient for efficient heterodimerization with Bcl-2 and Bcl-x<sub>L</sub>. Protein interaction studies further reveal that an 18-amino acid region, encompassing the BH3 domain (residues 57–74), constitutes the core heterodimerization domain. Functional analysis indicates that a Bik deletion mutant expressing residues 43–120, which efficiently heterodimerizes with the anti-apoptosis proteins Bcl-2 and Bcl-x<sub>L</sub>, is defective in eliciting cell death. In contrast, a mutant expressing additional C-terminal sequences (amino acids 43–134) interacts with the survival proteins and elicits efficient cell death. Our results suggest that for Bik-mediated cell death, the heterodimerization activity encoded by the BH3 domain alone is insufficient and raise the possibility that Bik may induce cell death autonomous of heterodimerization with survival proteins such as Bcl-2 and Bcl-x<sub>L</sub>.

Apoptosis is a normal physiological process required for selective elimination of cells in multicellular organisms. This process is either positively or negatively modulated by a number of external and internal factors. The bcl-2 proto-oncogene is a well known inhibitor of apoptosis (reviewed in Refs. 1 and 2). Recently, a number of vertebrate cellular and viral proteins function as dominant death inducers when overexpressed. These pro-apoptotic proteins promote cell death by complexing with and antagonizing the activity of endogenous survival proteins such as Bcl-2 and Bcl-x<sub>L</sub>. The pro-apoptotic homologs of Bcl-2 have been identified. The various Bcl-2 homologs have been shown to possess either anti-apoptotic or pro-apoptotic activity. The pro-apoptotic homologs include Bax (3), Bak (4–6), Bik (7, 8), Bid (9), and Hrk (10), which function as dominant death inducers when overexpressed. The mechanism(s) by which anti-apoptotic and pro-apoptotic proteins elicit their respective activities are not known. A common feature of the various Bcl-2 family members is that they form homo- and heterodimeric complexes (3, 11, 12). Based on this observation, it has been proposed that anti-apoptotic proteins such as Bcl-2 promote cell survival by complexing with pro-apoptotic proteins such as Bax, thereby antagonizing the death-promoting activity of these proteins (3, 11). However, it has recently been observed that certain mutants of Bcl-x<sub>L</sub>, defective in complex formation with Bax and Bak, are still able to promote cell survival (13). This raises the possibility that at least certain anti-apoptotic members of the Bcl-2 family of proteins may promote cell survival independently of their interaction with known pro-apoptotic proteins such as Bax and Bak.

The pro-apoptotic proteins Bax and Bak are closely related to Bcl-2 whereas Bik is more distantly related. Bax and Bak contain three conserved domains, BH1, BH2, and BH3, similar to Bcl-2, in addition to the characteristic C-terminal transmembrane domain common to all Bcl-2 family proteins. In contrast, Bik shares only the BH3 and the C-terminal transmembrane domains with other Bcl-2 family proteins. The presence of the BH3 domain in Bik, which is otherwise unrelated to Bax and Bak, suggests a critical role for the BH3 domain in the common cell death-inducing activity of the different proteins. The dual role of the BH3 domain in inducing cell death and mediating heterodimerization with survival proteins was elucidated by detailed mutagenesis of Bax, where a minigene encoding a 51-amino acid region, encompassing the BH3 domain, was found to be sufficient for inducing cell death and for heterodimerization with Bcl-x<sub>L</sub> (14). Further, mutations within the BH3 domain of Bak, Bax, and Bik were found to abrogate the ability of these proteins to heterodimerize with the survival proteins such as Bcl-2 and Bcl-x<sub>L</sub> and to eliminate their apoptotic activity (7, 14). Like Bik, two recently identified pro-apoptotic proteins, Bid (9) and Hrk (10), that complex with Bcl-2 and Bcl-x<sub>L</sub> share only the BH3 domain with other Bcl-2 family members. Thus, the two activities (i.e. heterodimerization with survival proteins and induction of cell death) of Bax, Bik, Bid, and Hrk mediated by the BH3 domain appear to be linked. These results are consistent with the hypothesis that these pro-apoptotic proteins promote cell death by complexing with endogenous survival proteins such as Bcl-2 and Bcl-x<sub>L</sub>. The results presented in this report suggest that complex formation of Bik with Bcl-2 and Bcl-x<sub>L</sub> alone is insufficient for promoting cell death. Efficient Bik-mediated cell death requires additional sequence elements, in addition to the conserved BH3 domain.

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Mutant Library—To construct N-terminal deletion mutants of Bik, the plasmid pACT-Bik (7) was linearized with BamHI, treated with Bal-31 nuclease (Life Technologies, Inc.) for various lengths of time, and then digested with SauI (3′ end of Bik cDNA). DNA fragments were isolated by agarose electrophoresis and ligated with the vector pACT (between BamHI and fragment ended with T4 DNA polymerase) and SauI sites. The ligated DNA was introduced into E. coli DH5α by electroporation. Transformed colonies (~10^5) were pooled by scraping and amplified for purification of library DNA. To construct C-terminal deletion mutants of Bik, pACT-Bik was linearized with SmaI (located at the 3′ end of the Bik open reading frame) and digested with Bal-31 and then with BamHI. DNA fragments were purified and ligated to pACT (between SauI/blunt ended and BamHI sites) and cloned.

Two-hybrid Analysis—Yeast indicator strain GGY1::171 was transformed with the bait plasmid expressing a fusion protein consisting of the human Bcl-2 protein tagged with the GAL4 DNA binding domain (pMA424-Bcl2; Ref. 15) and the GAL4 activation domain tagged with the Bik prey plasmid (pACT-Bik) and mutant library using established procedures (16, 17). DNA of the positive bik mutants were isolated and sequenced by fentomole cycle sequencing.

Immunoprecipitation Analysis—BSC40 or MCF-7 cells were infected with vaccinia virus vTF7–3 (10 plaque-forming units/cell) and cotransfected with 3 μg of each plasmid (pcDNA3-based) expressing Bcl-2 or Bcl-xL (18) and HA epitope-tagged Bik or Bik mutants. The cells were labeled with [35S]methionine mix (150 μCi/ml) in methionine-free medium (20–22 h postinfection) and lysed with TNN buffer (40 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The HA epitope-tagged proteins were immunoprecipitated and resolved by 15% SDS-PAGE electrophoresis. The expression of Bcl-2 was confirmed by immunoprecipitation with the anti-Bcl-2 antibody, 4D7 (Pharmingen) whereas Bcl-xL expression (tagged with the Flag epitope) was confirmed by immunoprecipitation with the anti-Flag antibody (Kodak).

Cell Death Assay—MCF-7 cells were transfected with HA epitope-tagged Bik or Bik mutants (2 μg) expressed from the pcDNA3 vector along with the reporter plasmid pRSV-β-gal (0.5 μg). After 9–10 h of transfection, the cells were fixed, stained with X-gal, and microscopically examined. 100–200 blue color cells were microscopically scored as flat (live) and round (apoptotic) cells.

RESULTS AND DISCUSSION

To determine the minimal sequences of Bik required for interaction with anti-apoptosis proteins, we used a library of N-terminal deletion mutants of Bik generated by Bal-31 nuclease digestion. The mutants competent for interaction with Bcl-2 were identified by two-hybrid analysis in yeast (13). Yeast colonies expressing the positively interacting bik mutants were identified on the basis of activation of expression of the lacZ reporter gene. The level of interaction was assessed from the intensity of blue color in the X-gal filter assay. Most of the yeast colonies resulting from transformation of this library and the Bcl-2 bait were white in the X-gal assay. However, two weakly interacting clones (which showed less intense blue color in the X-gal assay) were identified. DNA sequence analysis of these bik clones revealed that both clones had a single N-terminal end at codon 57 (Fig. 1f). These results indicate that residue 57 defines the N terminus of the minimal Bik protein required for heterodimerization with Bcl-2 whereas additional residues up to 43 amino acids are required for more efficient interaction.

To determine the C-terminal boundary, a similar Bal-31 nuclease-mediated deletion mutagenesis of pACT-Bik (wt) was performed, and the products were analyzed for interaction by two-hybrid analysis. Among the positively interacting clones, 18 were sequenced and 4 were chosen for further analysis. These clones had C-terminal ends at residues 96, 82, 74, and 74 (Fig. 1, g–j). Among the four mutants, mutant j (Fig. 1j) was interesting since it had the largest deletion and interacted with Bcl-2 better than mutant i. However, mutant j contained an additional 21 missense amino acids (unrelated to Bik, resulting from mutagenesis and cloning) at the C terminus compared with mutant i, which had only four missense amino acids. To determine if a truncation mutant of Bik with an additional deletion of the C-terminal sequences can interact with Bcl-2, a different mutant of pACT-Bik expressing the N-terminal 72 amino acids was constructed by site-directed mutagenesis and cloning into the Bik vector (pMA424-Bik with Bik residues 1–72). This truncation mutant was defective in interaction with Bcl-2, suggesting that an additional C-terminal sequence is required for Bik-induced apoptosis.

In summary, we found that Bik residues 57–74 may constitute the core heterodimerization motif and that additional flanking sequences are required to facilitate heterodimerization with Bcl-2.
deletion mutation (ΔGD) within the BH3 domain of Bik mutants 43–94 (Fig. 1o) and 43–120 (Fig. 1p) abolished their interaction with Bcl-2 underscoring the requirement of the BH3 domain for heterodimerization.

The ability of the three deletion mutants, 43–94, 43–120, and 43–134, to heterodimerize with cellular anti-apoptosis proteins Bcl-2 and Bcl-xL was investigated by coimmunoprecipitation analysis in mammalian cells. For this purpose, the wt Bik and mutants were tagged with the HA epitope and coexpressed with Bcl-2 or Bcl-xL in BSC40 cells using the vaccinia virus expression system (19). The proteins were immunoprecipitated with the HA antibody or the antibody specific for the survival proteins. In these studies, Bik mutants with deletions in the BH3 domain (ΔGD) were used as the negative control. As shown in Fig. 2A, Bcl-2 is coimmunoprecipitated with full-length HA-Bik as well as with the Bik deletion mutants HA-Bik(43–134) and HA-Bik(43–120) when the protein extracts were immunoprecipitated with the HA monoclonal antibody. Although the expression of the mutant HA-Bik-(43–94) was generally poor (compared with other mutants), it was also found to complex with Bcl-2 at detectable levels. It should be noted that expression of the various Bik mutants in the absence of Bcl-2 was generally lower than in cells expressing Bcl-2. It is possible the protein heterodimerization may stabilize the mutant proteins. As expected, there was no significant coprecipitation of Bcl-2 with the HA-BikΔGD (BH3) mutant, suggesting that the interaction is specific. Similarly, HA antibody did not precipitate Bcl-2 from cells that were not transfected with Bik expression vectors. These results indicate that all three deletion mutants of Bik can form a complex with Bcl-2 in mammalian cells and are in good agreement with the two-hybrid data. Similar coimmunoprecipitation studies were also carried out with extracts from cells transfected with Bcl-xL (tagged with Flag epitope) and Bik (Fig. 2B). In general, the relative level of interaction between Bik and Bcl-xL was stronger. Coexpression of HA-Bik wt or the various HA-Bik mutants with Bcl-xL resulted in highly efficient coprecipitation, and in contrast, coexpression with the BikΔGD mutant did not result in detectable amounts of Bcl-xL coprecipitation. Interestingly, coexpression of the Bik mutants with Bcl-xL resulted in high levels of Bik expression. These results indicate that all three
deletion mutants of Bik efficiently heterodimerize with Bcl-xL.

To determine if the two activities of Bik (i.e., ability to heterodimerize with survival proteins and to induce cell death) can be separated, we then determined the apoptotic activities of the three bik mutants by transient DNA transfection (14, 20) in MCF-7 cells. Expression of wt Bik resulted in about 80% apoptotic cells among the transfected cells (Fig. 3A). Similarly, expression of the mutant 43–134 induced cell death at levels comparable with that of wt Bik. In contrast, cells transfected with the corresponding mutant constructs containing the BH3 deletion (∆GD) mutation did not exhibit significantly higher frequency of cell death compared with cells transfected with the pcDNA3-HA vector. Interestingly, the heterodimerization-positive mutants 43–120 and 43–94 (as well as their derivatives containing the ∆GD mutation) were defective in induction of cell death. To resolve if the defect in induction of cell death by mutants 43–120 and 43–94 is not due to lack of mutant Bik protein expression in MCF-7 cells, we examined the expression of mutant proteins by immunoprecipitation analysis (Fig. 3B). Although mutant 43–94 was expressed at relatively low levels, mutant 43–120 was expressed at significant levels. Since cells transfected with the mutant 43–120 do not exhibit any higher cell death compared with cells transfected with the pcDNA3-HA vector, it appears that additional sequences encompassing residues 121–134 are essential for efficient Bik-mediated cell death.

The requirement of amino acids (aa) 121–134 for Bik-induced cell death was also confirmed using a different Bik mutant (∆121–134) containing an internal deletion of residues 121–134 within Bik-(43–160) (Figs. 4B and 5). A data bank search revealed that this region is significantly homologous to the substrate binding pocket (position P4) domain of Ced-3/ICE family caspases (22, 23) that mediate apoptosis (Fig. 4A). To determine the role of this motif in Bik-induced cell death, we introduced a deletion mutation (∆WW; residues 122–130) within the death-inducing Bik truncation mutant 43–134. Similarly, a different two-amino acid substitution mutant (R123A/S129A) was also constructed (Fig. 4B). The cell death activity of these mutants was determined by transient transfection. Deletion of the entire pocket motif impaired the death activity of Bik-(43–134) by about one-half, and mutation of the conserved Arg123 and Ser129 residues (R123A/S129A) resulted in modest reduction in the cell death activity compared with Bik-(43–134). These results suggest that the sequences encompassing residues 121–134 are required for efficient Bik-mediated cell death, and the sequence motif similar to the substrate binding pocket of Ced-3/ICE caspases may constitute part of these sequences.

We have utilized the yeast two-hybrid assay to identify minimal Bik sequences required for interaction with Bcl-2 from libraries of bik mutants. These studies indicate that a 52-amino acid region encompassing the BH3 domain of Bik is sufficient for heterodimerization with Bcl-2 and Bcl-xL. This result is in good agreement with the results obtained with two other cell death-inducing proteins Bak (14) and Bax (21). In the case of Bak, a 51-amino acid region (aa 73–123), and in the case of Bax, a 43-amino acid region (aa 59–101), both proteins encompassing the BH3 domain have been shown to be sufficient for heterodimerization with Bcl-xL and Bcl-2, respectively. Since our two-hybrid analysis indicates that a 18-amino acid region located between residues 57 and 74 mediates heterodimerization, the adjoining sequences may play a facilitating role. Recent in vitro peptide binding studies indicate that 16-amino acid regions encompassing the BH3 domains of Bak, Bax, and

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**FIG. 4.** Homology between Bik and the substrate recognition pocket motifs of cysteine proteases and Bik mutants in the C-terminal region. A, the sequences for the various Ced-3/ICE family caspases are from accession numbers U39613 (ICE-LAP3, caspase-7), U56390 (ICE-LAP6, caspase-9), U27463 (CPP-32, caspase-3) and L29052 (Ced-3). B, structures of Bik C-terminal mutants. Mutant ∆121–134 contains an internal deletion within Bik-(43–160) whereas other deletion and aa substitution mutants are within Bik-(43–134). TM, indicates transmembrane domain.

**FIG. 5.** Induction of apoptosis by Bik C-terminal mutants. A, cell death activity of Bik mutants. B, immunoprecipitation analysis of Bik C-terminal mutants. The apoptosis assays were carried out and quantitated as described in the legend to Fig. 3. 35S-Labeled proteins were immunoprecipitated from extracts of MCF-7 cells transfected with various HA-tagged Bik plasmids, immunoprecipitated with HA antibody, and analyzed by 15% SDS-PAGE and autoradiography.
Bik can bind with Bcl-xL (24). The results of these in vitro studies are in good agreement with the data from our two-hybrid analysis.

An important outcome of our present studies is the identification of mutants of Bik (e.g. Bik-(43–94) and Bik-(43–120)) that heterodimerize with cellular anti-apoptosis proteins Bcl-2 and Bcl-xL, and are unable to induce efficient cell death. Previous studies have indicated that the cell death activities of Bik, Bax, and Bik are closely linked with their ability to heterodimerize with survival proteins (7, 14). Similarly, mutations within the BH3 domain of two recently identified pro-apoptotic proteins Bid (9) and Hrk (10) impair their ability to heterodimerize with survival proteins and to induce cell death. These results are consistent with a model that the five different pro-apoptotic proteins may mediate cell death by complexing with endogenous survival proteins such as Bcl-2 and Bcl-xL.

Our present results indicate that interaction of Bik with anti-apoptosis proteins alone is insufficient for induction of cell death by Bik. Although we cannot rule out that the death-promoting activity of Bik is dependent on its interaction with cellular anti-apoptosis proteins, our results would be consistent with a model that Bik promotes cell death independently of such interaction.

Our present results also indicate that sequences of Bik encompassing a C-terminal region (aa 120–134) are required (in addition to the BH3 domain) for efficient cell death activity in MCF-7 cells. These sequences are not conserved in other pro-apoptotic members such as Bax, Bak, Bid, and Hrk. Since the BH3 domain plays a central role in mediating cell death by the various pro-apoptotic members, it is possible that in Bik, the C-terminal sequences may function in concert with the BH3 domain whereas in other pro-apoptotic proteins, the BH3 domain may function more autonomously or in concert with other yet unknown auxiliary sequence elements. The precise biochemical activity of the C-terminal region of Bik is not known. Although this region contains a sequence motif similar to the substrate binding motif of Ced-3/ICE family caspases, it remains to be determined if this motif modulates the substrate-caspase complexes. Simpler explanations such as the C-terminal region playing a role in Bik protein folding are also possible.

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