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harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X_L

Naohiro Inohara, Liyun Ding, Shu Chen and Gabriel Núñez[1]

Department of Pathology and Comprehensive Cancer Center, The University of Michigan Medical School, Ann Arbor, MI 48109, USA

[1]Corresponding author

Programmed cell death is essential in organ development and tissue homeostasis and its deregulation is associated with the development of several diseases in mice and humans. The precise mechanisms that control cell death have not been elucidated fully, but it is well established that this form of cellular demise is regulated by a genetic program which is activated in the dying cell. Here we report the identification, cloning and characterization of *harakiri*, a novel gene that regulates apoptosis. The product of *harakiri*, Hrk, physically interacts with the death-repressor proteins Bcl-2 and Bcl-X_L, but not with death-promoting homologs, Bax or Bak. Hrk lacks conserved BH1 and BH2 regions and significant homology to Bcl-2 family members or any other protein, except for a stretch of eight amino acids that exhibits high homology with BH3 regions. Expression of Hrk induces cell death which is inhibited by Bcl-2 and Bcl-X_L. Deletion of 16 amino acids including the conserved BH3 region abolished the ability of Hrk to interact with Bcl-2 and Bcl-X_L in mammalian cells. Moreover, the killing activity of this mutant form of Hrk (Hrk ΔBH3) was eliminated or dramatically reduced, suggesting that Hrk activates cell death at least in part by interacting with and inhibiting the protection afforded by Bcl-2 and Bcl-X_L. Because Hrk lacks conserved BH1 and BH2 domains that define Bcl-2 family members, we propose that Hrk and Bik/Nbk, another BH3-containing protein that activates apoptosis, represent a novel class of proteins that regulate apoptosis by interacting selectively with survival-promoting Bcl-2 and Bcl-X_L.

Keywords: apoptosis/Bcl-2 family/Hrk/programmed cell death

Introduction

Apoptosis, a morphologically distinguished form of programmed cell death, is critical not only during development and tissue homeostasis but also in the pathogenesis of a variety of diseases including cancer, autoimmune disease, viral infection and neurodegenerative disorders (Thompson, 1995). The precise mechanisms that regulate apoptosis have not been elucidated; however, it appears that this form of cell death is controlled by a genetic program which is activated in the dying cell (Thompson, 1995; White, 1996). Several regulatory components of the apoptotic pathway have been identified in various living organisms including man (Thompson, 1995; White, 1996). *bcl-2*, the first member of an evolutionarily conserved family of apoptosis regulatory genes, was isolated initially from the t(14;18) chromosomal translocation found in human B-cell follicular lymphomas and subsequently was shown to repress cell death triggered by a diverse array of stimuli (Vaux *et al.*, 1988; Núñez *et al.*, 1990; Strasser *et al.*, 1991). Several members of the family including Bcl-2, Bcl-X_L, Mcl-1, A1, Bcl-w and Ced-9 share conserved regions termed Bcl-2 homology domain 1, 2, 3 and 4 (BH1, BH2, BH3 and BH4) and function by repressing apoptosis (for review, see White, 1996). In contrast, other members of the family, such as Bax and Bak, share BH1, BH2 and BH3 domains and regulate apoptosis by promoting cell death at least in part through physical interactions with death-repressing Bcl-2 family members (Oltvai and Korsmeyer, 1994; Sato *et al.*, 1994).

Two members of the family, Bcl-2 and Bcl-X_L, are expressed in many embryonic and adult tissues, and their importance has been revealed by analysis of mutant mice deficient in Bcl-2 or Bcl-X_L. Null mutations of *bcl-x* are embryonic lethal with the occurrence of profound cell death, whereas mice deficient in *bcl-2* die early after birth (Veis *et al.*, 1993; Motoyama *et al.*, 1995). However, the biochemical process by which Bcl-2 and Bcl-X_L repress cell death and the mechanisms that regulate their functions are poorly understood. The susceptibility of a cell to apoptotic signals appears to be regulated in part by the relative levels and competing dimerizations of different Bcl-2 family members (Oltvai and Korsmeyer, 1994). In addition to Bcl-2 family members, Bcl-2 is known to interact with several cellular proteins that include R-Ras, Nip proteins and BAG-1 which appear to be part of signal transduction pathways (Boyd *et al.*, 1994; Fernandez-Sarabia and Bischoff, 1994; Takayama *et al.*, 1995; Wang *et al.*, 1996). However, the biochemical significance of these Bcl-2 interactions is unclear and poorly understood.

Here we report the identification, cloning and characterization of *harakiri*, a novel apoptosis regulatory gene. The product of *harakiri*, Hrk, physically interacts with the death-repressor proteins Bcl-2 and Bcl-X_L, but not with death-promoting Bax or Bak. Hrk lacks conserved BH1 and BH2 regions and significant homology to Bcl-2 family members or any other protein, except for a stretch of eight amino acids that exhibits high homology with BH3 regions. Hrk structurally resembles Bik/Nbk, another BH3-containing protein which was identified recently as an interacting partner of the adenovirus E1B 19K protein and Bcl-2 (Boyd *et al.*, 1995; Han *et al.*, 1996b). Beyond the conserved BH3 region, however, Bik/Nbk and Hrk do not share any significant amino acid homology. Expression of Hrk induced cell death which was repressed by Bcl-2 and

Bcl-X_L. Deletion of the conserved BH3 region in Hrk abolished its ability to interact with Bcl-2 and Bcl-X_L, and dramatically diminished its killing activity when compared with wild-type Hrk. Because Hrk lacks conserved BH1 and BH2 domains that define Bcl-2 family members, we propose that Bik/Nbk and Hrk represent a novel class of proteins that regulate apoptosis at least in part by interacting selectively with survival-promoting Bcl-2 and Bcl-X_L.

Results

Two-hybrid screening for proteins that bind to Bcl-2 reveals a novel protein that interacts with anti-apoptosis proteins Bcl-2 and Bcl-X_L but not with death-promoting proteins Bax and Bak

To search for Bcl-2-interacting proteins, we screened a HeLa cDNA library using GAL4–Bcl-2 as a 'bait' in the yeast two-hybrid assay. In a screen of 3×10^6 library clones, 30 positive clones were identified that interacted with the GAL4–Bcl-2 bait. Plasmids recovered from the original yeast strain were used in a co-transformation assay with the original GAL4–Bcl-2 bait or control heterologous baits to discard false-positive clones. Twenty one cDNAs (designated BP1–21) were found to contain inserts of different sizes (from 1.3 to 0.7 kb) that interacted with the GAL4–Bcl-2 bait but not with control baits. These cDNA clones were characterized by restriction enzyme mapping and nucleotide sequence analysis. As expected, multiple clones encoded proteins which are known to associate with Bcl-2 in mammalian cells. Seven cDNAs encoded Bad, three encoded Bax and two encoded Bik/Nbk (Oltvai *et al.*, 1993; Boyd *et al.*, 1995; Yang *et al.*, 1995; Han *et al.*, 1996b). Four cDNAs encoded a Ras-related protein (Yamagata *et al.*, 1994). The two-hybrid screen also yielded five cDNA clones with novel nucleotide sequences. Restriction enzyme and sequence analysis revealed that four of the novel positive clones contained identical inserts of 712 bp fused to the GAL4 DNA-binding domain. One of these four cDNA clones was characterized further. We have named this novel gene '*harakiri*' and the product that it encodes Hrk, after the Japanese suicide ritual (see below).

To characterize the specificity of the Hrk interactions further, we determined the ability of Hrk to interact with Bax, Bak and Bcl-X_S, three Bcl-2-related proteins that promote cell death. In the yeast two-hybrid system, Hrk interacted with Bcl-2 and Bcl-X_L but not with Bax, Bak or Bcl-X_S (Figure 1). These results indicate that Hrk selectively interacts with Bcl-2 family members such as Bcl-2 and Bcl-X_L which are functionally related in that they are capable of inhibiting apoptosis. Importantly, Hrk failed to associate with Bcl-X_S, an alternative form of Bcl-X, that lacks an internal region of 62 amino acids that is required for Bcl-X_L protective function (Boise *et al.*, 1993).

Hrk interacts with Bcl-2 and Bcl-X_L in mammalian cells

To confirm that Hrk associates with Bcl-2 and Bcl-X_L in mammalian cells, a 293T human kidney cell line was transiently co-transfected with expression plasmids producing a Flag-tagged Hrk protein and Bcl-2, Bcl-X_L or control plasmid. In these initial experiments, the entire *harakiri* sequence that was fused to the GAL4 DNA-binding domain was cloned into expression plasmids to produce Flag-Hrk. Immunoprecipitates were prepared using hamster anti-Bcl-2 monoclonal antibody and subjected to SDS–PAGE and immunoblotting using anti-Flag or Bcl-2 antibody. Immunoblotting with anti-Bcl-2 antibody revealed that Flag-Hrk was co-immunoprecipitated with Bcl-2 (Figure 2A). Control immunoblotting with anti-Bcl-2 antibody confirmed that Bcl-2 was immunoprecipitated in lysates of cells transfected with the *bcl-2* plasmid (Figure 2A). The Hrk–Bcl-2 interaction was specific in that it required co-expression of both Bcl-2 and Flag-Hrk, and was not detected when the lysate was immunoprecipitated with control antibody (Figure 2A). To verify the interaction, we performed reciprocal experiments using anti-Flag antibody to immunoprecipitate Flag-Hrk, followed by Western blot with anti-Bcl-2 antibody. In agreement with the reverse experiment, Bcl-2 co-immunoprecipitated specifically with Flag-Hrk (Figure 2B).

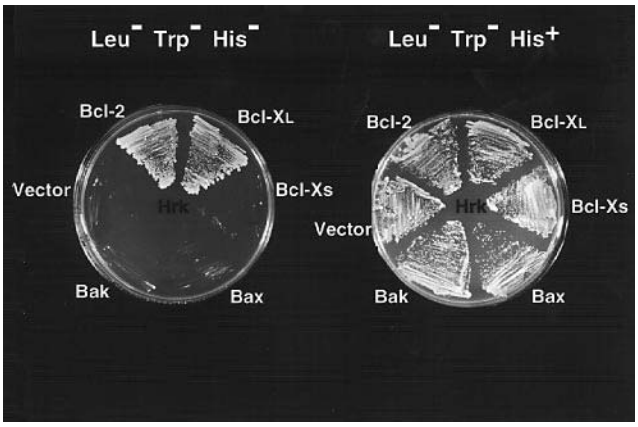

Fig. 1. Specificity of the interaction between Hrk and Bcl-2 family members in yeast. A plasmid expressing Hrk fused to the GAL4 DNA-binding domain was co-transfected with plasmids expressing Bcl-2 family members fused to the GAL4 transcriptional activation domain (AD). Growth of yeast in the absence of leucine, tryptophan and histidine is indicative of protein–protein interaction. Growth in the absence of leucine and tryptophan is shown as a control.

To determine if Hrk associates with Bcl-X_L in mammalian cells, we performed immunoprecipitation experiments similar to those performed to assess the Hrk–Bcl-2 interaction. After transfection of 293T cells with expression plasmids producing a Flag-tagged Hrk protein and Bcl-X_L or control plasmid, immunoprecipitates were prepared using anti-Bcl-X antibody and subjected to SDS–PAGE immunoblotting using anti-Flag antibody. Importantly, Flag-Hrk was co-immunoprecipitated specifically with Bcl-X_L (Figure 2C). To confirm the interaction, we performed reciprocal experiments using anti-Flag antibody to immunoprecipitate Flag-Hrk, followed by Western blot with anti-Bcl-X antibody. In agreement with the reverse experiment, Bcl-X_L co-immunoprecipitated specifically with Flag-Hrk (Figure 2D). We also tested if Hrk interacts with Bax in 293T cells and found that Flag-Hrk failed to co-immunoprecipitate with Bax, confirming our results in the yeast assay (data not shown).

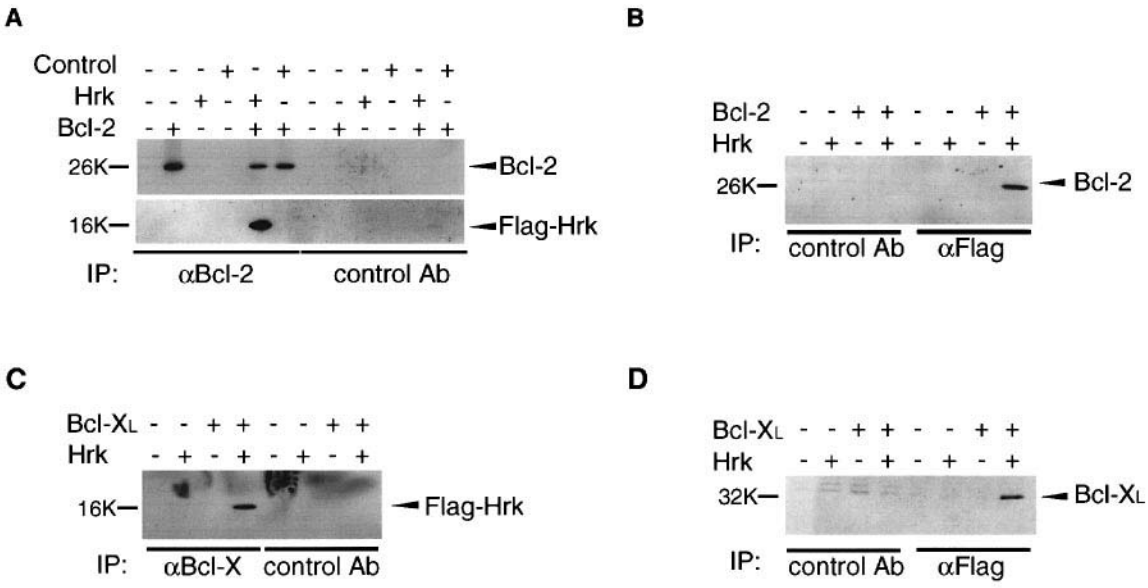

Fig. 2. Hrk interacts with Bcl-2 and Bcl-X_L in mammalian cells. 293T cells (5×10^6 per 100 mm plate) were transiently transfected with 10 μg of the indicated plasmids, pSFFV-Flag-tagged BP25 (control), pSFFV-Flag-Hrk16K (Hrk), pSFFV-hBcl-2 (Bcl-2), pSFFV-HA-hBcl-X_L (Bcl-X_L) or pSFFV-HA-mBax (Bax). In the case of transfection with a single plasmid, cells were co-transfected with 10 μg of empty pSFFV-neo vector and the total amount of plasmid DNA was always 20 μg. (**A**) Lysates were immunoprecipitated with anti-Bcl-2 or control antibody. Immunoprecipitates were immunoblotted with anti-Bcl-2 or anti-Flag antibody. (**B**) Anti-Flag or control antibody immunoprecipitates were immunoblotted with anti-Bcl-2 antibody. (**C**) Anti-Bcl-X or control antibody immunoprecipitates were immunoblotted with anti-Flag antibody. (**D**) Anti-Flag or control antibody immunoprecipitates were immunoblotted with anti-Bcl-X antibody. Notice that pSFFV-Flag-Hrk16K produces 16 kDa protein. as the entire *harakiri* cDNA including its 5′ untranslated region was fused in-frame with the Flag sequence and the amino-terminus of Hrk.

harakiri encodes a novel protein with a conserved BH3 region but lacking BH1 or BH2 domains

To confirm the sequence obtained by the two-hybrid assay, a 9-week embryo human cDNA library was screened by hybridization with a labeled *harakiri* probe. Forty positive cDNA clones were identified and characterized by restriction mapping, PCR analysis and sequencing. Analysis of the longest cDNAs revealed inserts of 716 bp essentially identical in sequence to that observed previously fused to the GAL4 DNA-binding domain. A single nucleotide difference C→T at position 334 of the *harakiri* cDNA was noted between the coding sequences obtained from the HeLa and embryo cDNA libraries (Figure 3A). This nucleotide difference did not change the amino acid sequence of Hrk, reflecting perhaps a gene polymorphism. Northern blot analysis of mouse and human tissues using a labeled *harakiri* probe identified a major mRNA species of ~0.7 kb in certain tissues (see below). Taken together, these results suggest that the isolated cDNA clones are full-length. The cDNAs encode an open reading frame of 91 amino acids (Figure 3A). In the 3′ untranslated region, an ATTTA sequence motif for RNA destabilization (Shaw and Kamen, 1986) and a poly(A) tail were identified. Initial DNA searches using the NCBI BLAST program revealed that the nucleotide sequence was novel in that they did not reveal significant homology to any known gene or translated products in the databases. However, close inspection of the Hrk protein revealed a stretch of eight amino acids, Hrk 37–44, with high homology to a BH3 motif. This conserved region is shared by Bcl-2 family members (Chittenden *et al.*, 1995b; Zha *et al.*, 1996). The BH3 region of Hrk was contained within a predicted α-helix which has been described recently in the crystal structure of Bcl-X_L (Muchmore *et al.*, 1996), and predicted to be present in other Bcl-2 family members (Figure 3C). A region of 28 hydrophobic residues that may serve as a membrane-spanning domain was identified at the COOH-terminus of the Hrk coding region (Figure 3A).

harakiri displays a highly restricted expression in human tissues

To characterize *harakiri* further, its expression pattern was examined by Northern blot analysis using a *harakiri* probe of various RNA samples isolated from human adult tissues. A major 0.7 kb transcript was detected in all lymphoid tissues tested but was expressed predominantly in bone marrow and spleen (Figure 4). Further analysis showed that *harakiri* mRNA was also expressed in pancreas and at very low or undetectable levels in kidney, liver, lung and brain (data not shown).

The Flag-Hrk protein is a non-nuclear intracellular protein

In order to begin to assess the subcellular localization of Hrk, we transiently transfected a Flag-*harakiri* construct into 293 cells and determined the intracellular localization of the Flag-Hrk protein by laser scanning confocal microscopy. Analysis of labeled cells with anti-Flag monoclonal antibody revealed that Flag-Hrk displays a labeling pattern which was granular and extranuclear, consistent with a localization confined to membranes of intracellular organelles (Figure 5A). The labeling pattern of Hrk was similar to that previously reported for Bcl-2 and Bcl-X_L, suggesting that Hrk, Bcl-2 and Bcl-X_L localize to similar intracellular compartments in mammalian cells (Krajewski *et al.*, 1993; González-García *et al.*, 1994). The specificity of the labeling was determined by assessing the staining

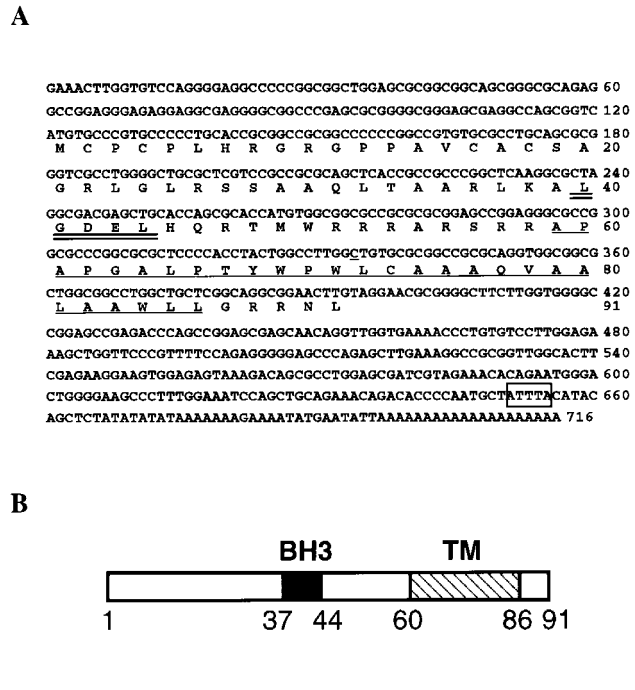

Fig. 3. *harakiri* encodes a novel BH3-containing protein. (**A**) The nucleotide sequence of human *harakiri* cDNA. The sequence from the longest cDNA clone isolated from a 9-week embryo is shown. The coding region is indicated with its amino acid sequence. A conserved BH3 region and a putative transmembrane domain are indicated by double and single underlining in the amino acid sequence, respectively. An ATTTA sequence motif for RNA destabilization identified in the 3′ untranslated region is boxed. A putative nucleotide polymorphism was observed, C→T at position 334 of the cDNA, and is shown by underlining in the nucleotide sequence. (**B**) Schematic structure of Hrk. (**C**) Comparison of the BH3 domains between Hrk and Bcl-2 family members. Identical and conserved hydrophobic residues are indicated by bold letters and stars, respectively. References for hBax, hBcl-2, hBcl-X, Bik/Nbk, Bak and Mcl-1 proteins are given in the Introduction.

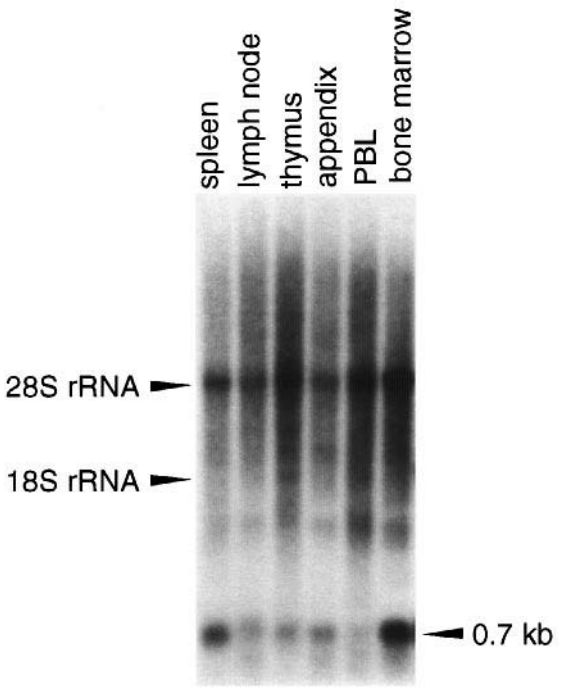

Fig. 4. Expression of *harakiri* mRNA by Northern blot analysis. A filter loaded with 2 μg of poly(A) RNA from various tissues of the immune system was hybridized with the entire *harakiri* cDNA probe labeled with ^{32}P, washed with 0.2× SSC at 65°C for 1 h. The filter was exposed to X-ray film for 12 h. 28S rRNA, 18S rRNA and the major transcript of 0.7 kb are indicated by arrowheads. PBL, peripheral blood leukocytes.

pattern obtained after transfection with a control Flag-tagged GATA-1 expression plasmid. Staining of the GATA-1 protein with anti-Flag antibody revealed a nuclear labeling pattern, as expected for a nuclear transcriptional factor (Figure 5B).

Expression of Hrk induces rapid cell death which is repressed by Bcl-2 and Bcl-X_L

The effect of *harakiri* on cell survival was examined initially in 293T cells using a transient transfection assay. Transfection of a *harakiri* expression plasmid into 293T cells resulted in a dramatic loss of cell viability at 36 h post-transfection (Figure 6B and F). This was specific in that transfection of 293T cells with empty vector, *bcl-2* or *bcl-x_L* expression plasmids did not have any effect on cell survival (Figure 6A, C, E and G). Significantly, co-expression of Bcl-2 or Bcl-X_L inhibited the death-promoting activity of Hrk (Figure 6D and H). To verify these results further, we performed additional experiments in which Hrk plasmids were transfected into 293, HeLa and FL5.12 progenitor B cells. Transient transfection of 293 and HeLa cells with Hrk plasmids induced cell death with kinetics similar to those observed with 293T cells (data not shown). To date, we have been unable to generate any stable FL5.12 cell lines that express transfected Hrk in the absence of exogenous Bcl-2 or Bcl-X_L, suggesting that Hrk expression is also lethal to these cells (data not shown).

Hrk requires a region of 16 amino acids containing BH3 to interact with Bcl-2 and Bcl-X_L

To determine the ability of Hrk to associate with Bcl-2 or Bcl-X_L through BH3, we engineered a mutant form of Hrk lacking BH3. A deletion mutant was designed to eliminate residues 34–49 of Hrk, a predicted α-helix, to minimize secondary effects on folding of the Hrk protein. The deleted residues contained the most conserved amino acids of the BH3 homology region of Hrk (Figures 3C and 7A). Expression plasmids producing wild-type or mutant Flag-tagged Hrk and Bcl-2 or Bcl-X_L were transiently co-transfected into 293T cells. Immunoprecipitates were prepared using anti-Flag to recognize wild-type and mutant Flag-Hrk and subjected to SDS–PAGE and immunoblotting using anti-Bcl-2 and anti-Bcl-X antibodies. Deletion of residues 34–49 (Hrk mutant ΔBH3) completely eliminated the ability of Hrk to interact with

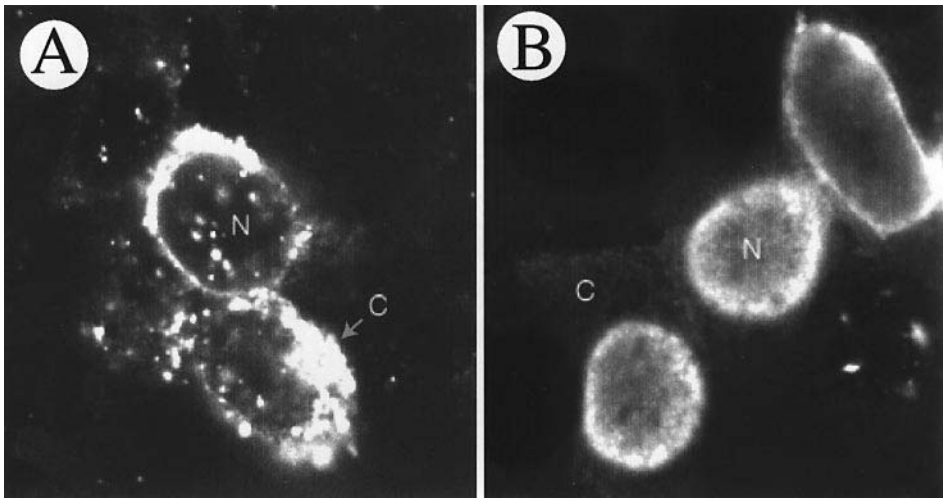

Fig. 5. Flag-Hrk protein is a non-nuclear intracellular protein. 293 cells (5×10^6 cells per 100 mm plate) were transfected with 10 μg of pSFFV-Flag-Hrk16K (**A**) or Flag-GATA-1 (**B**) as a control. Shown are confocal images after labeling with anti-Flag and secondary fluorescein-conjugated antibody. Samples were prepared at 18 h after transfection. Nuclei and cytoplasm are indicated by N and C, respectively. Notice that cells transfected with pSFFV-Flag-Hrk16K round up. Scattered bright particles probably represent cellular fragments from dying neighboring cells.

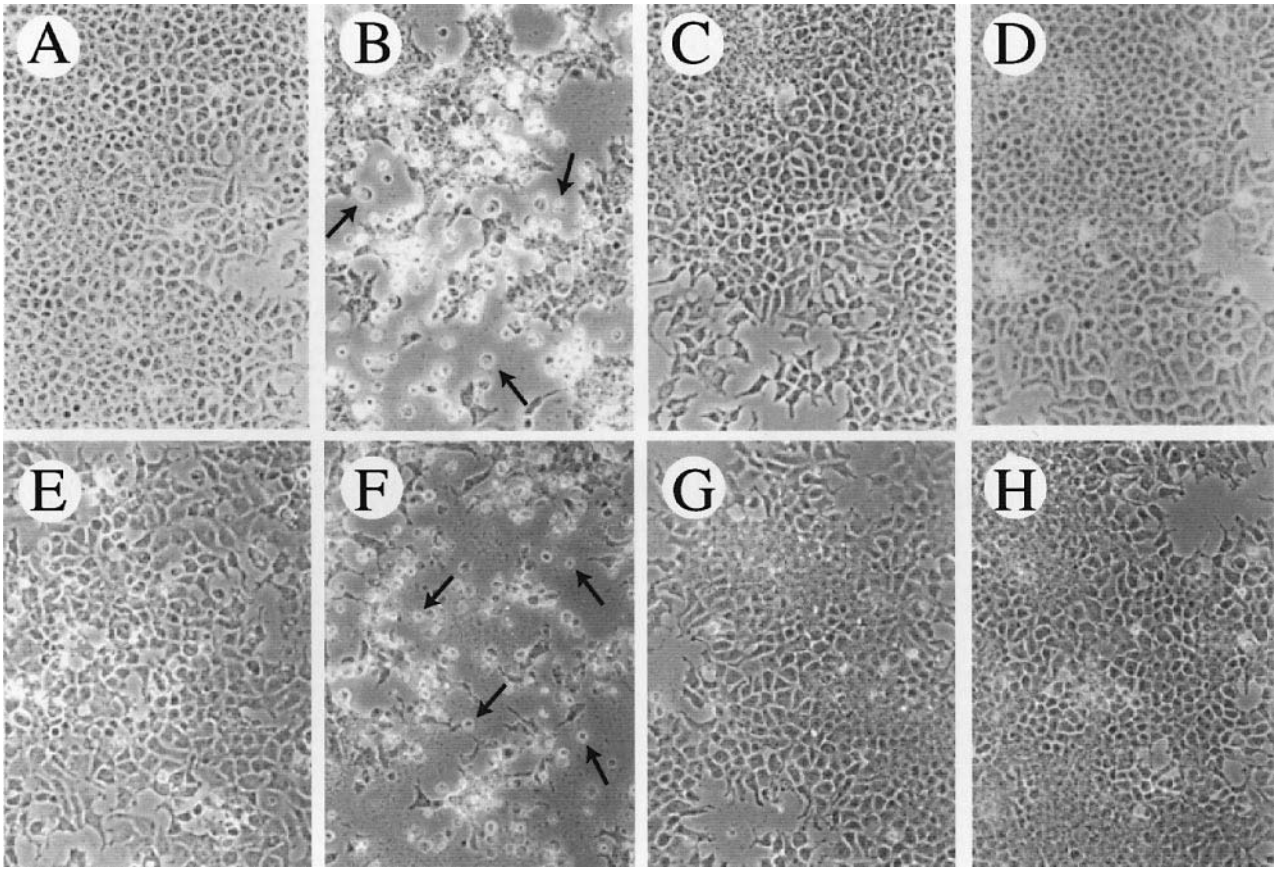

Fig. 6. Expression of Hrk induces cell death which is repressed by Bcl-2 or Bcl- X_L. 293T cells (5×10^6 per 100 mm plate) were co-transfected with 20 μg of pSFFV-neo (**A** and **E**), 10 μg of pSFFV-Flag-Hrk16K plus 10 μg of pSFFV-neo (**B** and **F**), 10 μg of pSFFV-hBcl-2 plus 10 μg of pSFFV-neo (**C**), 10 μg of pSFFV-Flag-Hrk16K plus 10 μg of pSFFV-hBcl-2 (**D**), 10 μg of pSFFV-HA-hBcl-X_L and 10 μg of pSFFV-neo (**G**), 10 μg of pSFFV-Flag-Hrk16K plus 10 μg of pSFFV-HA-hBcl-X_L (**H**) as described in Figure 2. Photographs represent cells at 36 h after transfection. Notice that cultures transfected with Flag-*harakiri* show a paucity of attached live cells and shrunken dead cells (arrows) which are blocked by co-transfection with *bcl-2* or *bcl-x_L*.

Bcl-2 and Bcl-X_L (Figure 7B). To confirm these results, we performed reciprocal experiments using anti-Bcl-2 antibody, followed by Western blot with anti-Flag antibody. In agreement with the reverse experiment, wild-type Hrk but not mutant Hrk co-immunoprecipitated specifically with Bcl-2 (Figure 7C). Analysis of total lysates from the

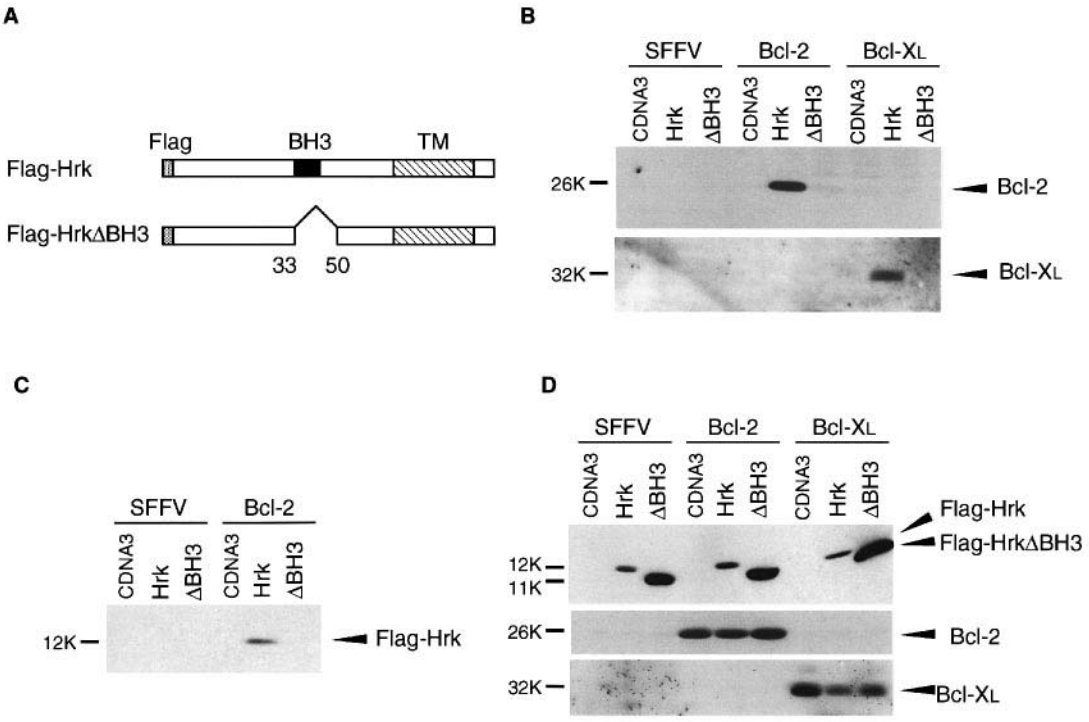

Fig. 7. Hrk requires a region of 16 amino acids containing BH3 to interact with Bcl-2 and Bcl- X_L. (**A**) Schematic structure of wild-type (Flag-Hrk) and mutant (Flag-Hrk ΔBH3) proteins. (**B**) 293T cells (5×10^6 per 100 mm plate) were co-transfected with 2 μg of pCDNA3 (CDNA3), pCDNA3-Flag-Hrk (Hrk) or pCDNA3-Flag-Hrk ΔBH3 (Flag-Hrk ΔBH3) and 10 μg of pSFFV-neo (SFFV), pSFFV-hBcl-2 (Bcl-2) or pSFFV-HA-hBcl-X_L (Bcl-X_L) as described in Figure 1. Anti-Flag immunoprecipitates were immunoblotted by anti-Bcl-2 (upper) and anti-Bcl-X antibody (lower). (**C**) Anti-Bcl-2 immunoprecipitates were immunoblotted with anti-Flag antibody. (**D**) Twenty μg of total lysate were immunoblotted with anti-Flag, anti-Bcl-2 or anti-Bcl-X antibody.

same cellular extracts by immunoblotting confirmed that 293T cells transfected with the corresponding plasmids expressed Bcl-2, Bcl-X_L, wild-type and mutant Hrk proteins (Figure 7D).

The BH3 domain of Hrk is required for the induction of cell death

To determine if the BH3 domain of Hrk is necessary to induce cell death, we compared the killing activity of wild-type and mutant Hrk proteins by a transient transfection assay in 293T cells. The cells were co-transfected with a reporter plasmid expressing GFP (green fluorescence protein), in combination with either an expression plasmid encoding Hrk proteins or control plasmids. The cell killing activity of wild-type and mutant Hrk was measured by a reduction in the number of cells that express the reporter GFP protein relative to that obtained by transfection with a control expression plasmid. The results of these experiments showed that deletion of residues 34–49 of Hrk (Hrk mutant ΔBH3) dramatically reduced the ability of Hrk to kill 293T cells, when compared with the activity exhibited by wild-type Hrk (Figure 8A). Furthermore, expression of Bcl-2 or Bcl-X_L inhibited the killing activity of wild-type Hrk, confirming the results presented in Figure 6. The loss of viability and the morphology of the cells observed after transfection with Hrk plasmids in Figure 6 suggested that Hrk may induce apoptotic cell death. To determine if the cell death activated by transient Hrk expression was caused by apoptosis, the nuclei of 293T cells were stained with acridine arange and ethidium bromide. Cells transfected with wild-type Hrk but not with mutant Hrk ΔBH3 nor control plasmid displayed nuclear fragmentation, a cytological change associated with apoptosis (Figure 8B).

Discussion

We have identified *harakiri*, a novel regulator of apoptosis that exhibits death-inducing activity in mammalian cells. Hrk interacts with Bcl-2 and Bcl-X_L, two death-repressing Bcl-2 family members that play essential roles in maintaining cell survival in embryonic and adult tissues (Veis *et al.*, 1993; Motoyama *et al.*, 1995). Hrk shares homology with Bcl-2 in the BH3 domain but, unlike most other Bcl-2 family members, it lacks conserved BH1 and BH2 regions. The BH3 region appears to represent a critical domain for interaction with Bcl-2 family members and regulation of apoptosis. For example, BH3 is required for death-promoting Bax and Bak to associate with Bcl-2 and Bcl-X_L (Chittenden *et al.*, 1995a; Han *et al.*, 1996a; Simonian *et al.*, 1996; Zha *et al.*, 1996). Moreover, deletion of BH3 prevented the killing activity of Bak and expression of a 30 amino acid region of Bak containing BH3 linked to a membrane anchor sequence was sufficient for cell killing activity in transient assays (Chittenden *et al.*, 1995b). In our studies, a 16 amino acid region of Hrk encompassing BH3 was required for Hrk to interact with Bcl-2 and Bcl-X_L, and its deletion eliminated or greatly reduced the killing activity of Hrk. In addition, these studies demonstrate that a region of 62 residues containing the BH1 and BH2 regions in Bcl-X_L is essential for its binding to Hrk since Bcl-X_S, an alternative form of

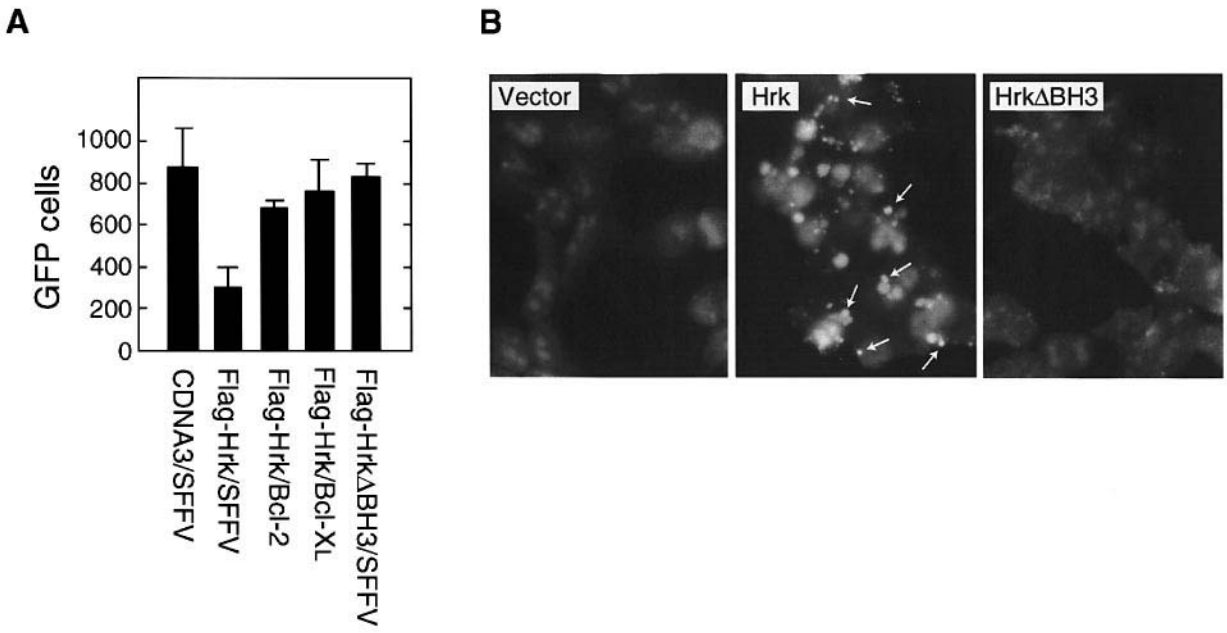

Fig. 8. The BH3 domain of Hrk is required for the induction of cell death. (**A**) 293T cells (1.7×10^6 per 35 mm plate) were transfected with 0.3 μg of pRK7.GFP and 0.8 μg of pCDNA3 (control), 0.8 μg of pCDNA3-Flag-Hrk (Hrk) or 0.8 μg of pCDNA3-Flag-Hrk ΔBH3 (ΔBH3) in the presence or absence of 6.7 μg of pSFFV-neo, pSFFV-hBcl-2 (Bcl-2) or pSFFV-HA-hBcl-X_L (Bcl-X_L) in triplicate. The number of live cells expressing GFP was determined at 48 h after transfection by analysis of 5×10^3 cells as described in Materials and methods. The results are shown as the mean of triplicate values ± SD. (**B**) 293T cells transfected with 0.8 μg of pCDNA3 (vector), pCDNA3-Flag-Hrk (Hrk) and pCDNA3-Flag-Hrk ΔBH3 (Hrk ΔBH3) were stained at 18 h after transfection with acridine orange and ethidium bromide to stain nuclei. Notice that nuclei of cells transfected with pCDNA3-Flag-Hrk were condensed and some of them are fragmented (arrows). In the experiment shown, ~20% of the transfected cells exhibited apoptotic changes in the nuclei.

Bcl-X lacking these conserved domains, failed to associate with Hrk.

Susceptibility of a cell to apoptotic signals appears to be regulated in part by the relative levels and competing dimerizations of death-suppressing and death-promoting Bcl-2 family members (Oltvai and Korsmeyer, 1994; Sato *et al.*, 1994). How could Hrk activate cell death? Two non-exclusive models could be proposed to explain the role of Hrk in cell death. Hrk could be an effector molecule with intrinsic death-inducing activity, and death-suppressing Bcl-2 and Bcl-X_L may serve as dominant inhibitors. In support of this model, overexpresssion of Bcl-2 or Bcl-X_L inhibited the killing activity of Hrk. Alternatively, Hrk could promote cell death by inhibiting the protective activity of Bcl-2 and Bcl-X_L and perhaps other functional homologs. Inactivation of Bcl-2 and Bcl-X_L would require the interaction of these survival proteins with Hrk and be mediated by the formation of Bcl-2–Hrk and/or Bcl-X_L–Hrk complexes. The analysis of the Hrk ΔBH3 mutant strongly supports the latter model in that a region of 16 residues containing the BH3 domain required for Hrk to interact with Bcl-2/Bcl-X_L was also necessary for Hrk killing activity. However, these studies cannot rule out that Hrk could also act as a death effector molecule as it is formally possible that the 16 amino acid deletion in Hrk directly or indirectly also affects an intrinsic death-inducing activity. Similarly, deletion mutant analysis of Bak has shown that elimination of BH3 greatly diminished the killing activity of Bak (Chittenden *et al.*, 1995b). Thus definitive evidence for the second model will require additional mutational studies and analysis of cells deficient in Hrk.

Oncogenic signals such as deregulated c-Myc or adenovirus E1A can promote or induce apoptosis in cell lines (Askew *et al.*, 1991; White *et al.*, 1991; Evan *et al.*, 1992). Thus, it is conceivable that in tumor cells, apoptotic signals are constitutively expressed, although repressed by Bcl-2 and/or Bcl-X_L. Consistent with this is the observation that expression of Bcl-X_S, a functional inhibitor of Bcl-2 and Bcl-X_L (Boise *et al.*, 1993), activates apoptosis in a wide variety of cancer cells (Clarke *et al.*, 1995). Thus, physical inactivation of Bcl-2 and Bcl-X_L by Hrk could similarly unleash endogenous death signals leading to execution of the apoptotic program. This hypothesis is consistent with recent observations in mutant mice lacking Bcl-2 and Bcl-X (Veis *et al.*, 1993; Motoyama *et al.*, 1995). Mice deficient in Bcl-X developed massive apoptosis of neural and hematopoietic tissues and died at E12–13 of embryonic development (Motoyama *et al.*, 1995), whereas newly born Bcl-2-deficient mice exhibited fulminant apoptosis of lymphoid tissues (Veis *et al.*, 1993). Thus, in addition to cancer cells, expression of Bcl-X or Bcl-2 appears necessary to counter death signals that arise during normal development. The precise mechanism of death triggered by Hrk needs to be determined, but we hypothesize that it involves the activation of interleukin 1-β converting enzyme-like proteases, a step which appears to be downstream of Bcl-2 and Bcl-X_L in the death pathway (Chinnaiyan *et al.*, 1996).

harakiri is the second member of a class of cell death regulatory genes that also include *bik*/*nbk*. Unlike 'classical' *bcl-2* family members, the proteins that they encode lack conserved BH1, BH2 or BH4 regions but share the BH3 domain. Our studies indicate that the BH3

domain of Hrk is critical for its interaction with Bcl-2 and Bcl-X_L. Although Hrk and Bik/Nbk share both a BH3 domain and a hydrophobic region at the COOH-terminus predicted to mediate attachment to intracellular membranes, these proteins are distinct and do not share additional amino acid homology. Thus, it is possible that there are subtle differences in the function of Hrk and Bik/Nbk. In this respect, it has been shown that Bcl-X_S interacts with Bik/Nbk (Boyd *et al.*, 1995), but our analysis revealed that Bcl-X_S fails to associate with Hrk. In addition, Hrk and Bik/Nbk differ in their pattern of expression in tissues (Boyd *et al.*, 1995), which suggests that they play distinct roles in the regulation of apoptosis *in vivo*. Future studies need to address a role for *harakiri* in the regulation of physiological cell death during tissue development and homeostasis.

Materials and methods

Screening for Bcl-2-interacting proteins by the yeast two-hybrid system

A HeLa cDNA library fused to the GAL4 activation domain of the pGAD-GH vector (Hannon *et al.*, 1993) was screened for proteins that interact with human Bcl-2, using the HF7c yeast reporter strain (Feilotter *et al.*, 1994). Briefly, the pGAD library plasmid was transformed into HF7c yeast cells harboring the pGBT-9-bcl-2 bait plasmid by standard transfection procedures. Transformed HF7 cells were plated on medium lacking tryptophan, leucine and histidine. A total of 3×10^6 library clones were screened for growth in selection medium and assayed for β-galactosidase activity. Positive clones were picked and yeast plasmid DNA from individual clones was used to transform *Escherichia coli* HB101 (leuB$^-$) cells. Library plasmids were recovered selectively from bacterial colonies by growth in media lacking leucine. False-positive clones were eliminated by testing for interaction with empty vector pGBT-8 and irrelevant 'baits'. cDNA inserts in the plasmid were characterized by restriction enzyme mapping and nucleotide sequence analysis using an automated DNA sequencer (Applied Biosystems Model 373A).

Plasmid construction

Plasmids expressing hBcl-X_L or hBcl-X_S (Boise *et al.*, 1993), mBax (Oltvai *et al.*, 1993) and hBak (Farrow *et al.*, 1995) in pGBT-9 were constructed by PCR amplification of plasmid cDNA to incorporate restriction sites, followed by ligation of the amplified DNA fragments in-frame with the GAL4 DNA-binding domain of pGBT-9. The authenticity of the GAL4 fusion plasmids was confirmed by dideoxy sequencing. The mammalian expression plasmids SFFV-Flag-hBcl-2, SFFV-Flag-hBcl-x_L and SFFV-Flag-mBax have been described (Merino *et al.*, 1995; Simonian *et al.*, 1996). Three different constructs were generated to express *harakiri* sequences. In an initial construction, a Flag epitope sequence was attached to nucleotide 7 of the 5′ untranslated region of the *harakiri* cDNA to generate Flag-Hrk16K by PCR using pGAD-GH-*harakiri* plasmid as a template (see Figure 3A). The Flag sequence in Hrk16K was in-frame with the amino-terminus of Hrk, resulting in a fusion protein with a predicted size of 16 kDa. In a second construction, a Flag-Hrk insert was constructed by introducing a Flag epitope tag at the amino-terminus of Hrk by PCR. A deletion of amino acids 34–49 that includes the BH3 domain of Hrk was generated by a two-step PCR mutagenesis method as described (Simonian *et al.*, 1996). A Flag epitope tag was attached to the amino-terminus of mutated Hrk protein to generate Flag-Hrk ΔBH3. A Flag epitope tag was attached to the amino-terminus of a control cDNA clone BP25 (N.Inohara, L.Ding and G.Núñez, unpublished data) by PCR. The Flag-tagged inserts were ligated into the *Eco*RI cloning site of pSFFV-neo or the *Bam*HI and *Xho*I sites of pCDNA3 (Invitrogen, San Diego, CA). Orientation of the inserts was determined by restriction mapping. The authenticity of all Flag-tagged constructs was confirmed by dideoxy sequencing. An expression plasmid that produces a Flag-tagged GATA-1 protein was obtained from Dr Vishva Dixit (Department of Pathology, University of Michigan).

Screening of human cDNA library by hybridization with labeled probe

A 9-week human embryo λgt11 cDNA library (Swaroop and Xu, 1993) was screened by hybridization with a ^{32}P-labeled cDNA containing the entire coding region of *harakiri*. Approximately 1×10^6 cDNA clones were screened by standard procedures, and positive phage clones were purified by sequential plating and hybridization. DNA inserts were characterized by PCR amplification of phage DNA from purified plaques, restriction enzyme mapping of phage DNA inserts and dideoxy sequencing.

Transfection, immunoprecipitation and Western blot analysis

Human embryonic kidney 293T and 293 cells were obtained from Dr Vishva Dixit (Department of Pathology, University of Michigan). Culture dishes containing cells were transfected with the indicated amount (see figure legends) of plasmid DNA by the calcium phosphate method. The expression of Flag-Bcl-X_L, Flag-Bcl-2, Flag-Hrk, Flag-Hrk ΔBH3 and Bax was determined in total lysates by Western blot analysis as previously described (Merino *et al.*, 1995; Simonian *et al.*, 1996). For immunoprecipitations, 1×10^7 cells were lysed in NP-40 isotonic lysis buffer (Oltvai *et al.*, 1993) at 38 h after transfection and the lysates were rotated with 10 μg/ml of anti-Flag M2 monoclonal antibody (Scientific Imaging Systems, Rochester, NY), hamster anti-Bcl-2 monoclonal antibody (Hockenbery *et al.*, 1991), rabbit anti-Bcl-X (Boise *et al.*, 1995) or control Ig overnight at 4°C. Then 5% (v/v) of protein A–Sepharose 4B (Zymed Laboratories Inc., San Francisco, CA) was added for an additional hour of incubation by rotation. Immune complexes were centrifuged and washed with excess cold NP-40 isotonic lysis buffer at least four times, separated on a 15% SDS–polyacrylamide gel and immunoblotted with anti-Bcl-2, anti-Flag, rabbit anti-Bcl-X or control Ig.

Northern blot analysis

A 716 bp *harakiri* cDNA was radiolabeled by the random priming method using a commercial kit (Boehringer Mannheim, Indianapolis, IN) and applied for analysis of human multiple tissue blots (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions.

Laser scanning confocal microscopy and fluorescence staining of nuclear DNA

293 cells were transfected with pCDNA3-Flag-Hrk, pCDNA3-Flag-GATA-1, or empty vector as described above. Twenty four hours after transfection, cells were incubated with anti-Flag monoclonal antibody or control mouse Ig for 1 h at 23°C and the labeling visualized with fluorescein-conjugated goat anti-mouse IgG. After washing, the cells were mounted in Slowfade (Molecular Probes, Eugene, OR) and examined using a BioRad MRC 600 scanning confocal microscope equipped with an argon–xenon laser (González-García *et al.*, 1994). Staining of nuclei with acridine orange and ethidium bromide was performed as described previously (Duke and Cohen, 1994).

Cell death assays

293T, 293 and HeLa cells were co-transfected by a calcium phosphate method with pRK7.GFP, a reporter plasmid expressing GFP (a gift of Roger Y.Tsien), in combination with an expression plasmid encoding Hrk, Bcl-2, Bcl-X_L or control plasmid. The number of plasmids transfected is indicated in the figure legends. Killing activity was determined at 48 h after transfection and based on the analysis of 5×10^3 cells by FACScan flow cytometry (Becton Dicknson, Mountain View, CA). In the assay, the cell killing activity was manifested by a reduction in the number of cells that express GFP relative to that obtained by transfection with the control expression plasmid.

Accession number

The accession number for the nucleotide sequence of *harakiri* cDNA reported in this paper is U76376.

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References

Askew,D., Ashmun,R., Simmons,B. and Cleveland,J. (1991) Constitutive c-*myc* expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene*, **6**, 1915–1922.

Boise,L.H., González-García,M., Postema,C.E., Ding,L., Lindsten,T., Turka,L.A., Mao,X., Núñez,G. and Thompson,C.B. (1993) *bcl-x*, a *bcl-2* related gene that functions as a dominant regulator of apoptotic cell death. *Cell*, **74**, 597–608.

Boise,L.H., Minn,A.J., Noel,P.J., June,C.H., Accavitti,M.A., Lindsten,T. and Thompson,C.B. (1995) CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-X_L. *Immunity*, **3**, 87–98.

Boyd,J.M., Malstrom,S., Subramanian,T., Venkatesh,L.K., Schaeper,U., Elangovan,B., D'Sa-Eipper,C. and Chinnadural,G. (1994) Adenovirus E1B and 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. *Cell*, **79**, 341–351.

Boyd,J.M. *et al*. (1995) Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene*, **11**, 1921–1928.

Chinnaiyan,A.M., Orth,K., O'Rourke,K., Duan,H., Poirier,G.G. and Dixit,V.M. (1996) Molecular ordering of the cell death pathway: Bcl-2 and Bcl-X_L function upstream of the CED-3-like apoptotic proteases. *J. Biol. Chem*., **271**, 4573–4576.

Chittenden,T., Harrington,E.A., O'Connor,R., Flemington,C., Lutz,R.J., Evan,G.I. and Guild,B.C. (1995a) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature*, **374**, 733–736.

Chittenden,T., Flemington,C., Houghton,A.B., Ebb,G.E., Gallo,G.J., Elangovan,B., Chinnadurai,G. and Lutz,R.J. (1995b) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J*., **14**, 5589–5596.

Clarke,M.F. *et al*. (1995) A recombinant *bcl-*x_s adenovirus selectively induces apoptosis in cancer cells, but not normal bone marrow cells. *Proc. Natl Acad. Sci. USA*, **92**, 11024–11028.

Duke,R.C. and Cohen,J.J. (1994) Morphological and biochemical assays of apoptosis. In Coligan,J.E., Kruisbeek,A.M., Margulies,D.H., Shevach,E.M. and Strober,W. (eds), *Current Protocols in Immunology*. Current Protocols, NY, pp. 3.17.1–3.17.3.

Evan,G.I., Wyllie,A.H., Gilbert,C.S., Littlewood,T.D., Land,H., Brooks,M., Waters,C.M., Penn,L.Z. and Hancock,D.C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell*, **69**, 119–128.

Farrow,S.N., White,J.H., Martinou,I., Raven,T., Pun,K.T., Grinham,C.J., Martinou,J.C. and Brown,R. (1995) Cloning of a *bcl-2* homologue by interaction with adenovirus E1B 19K. *Nature*, **374**, 731–733.

Feilotter,H.E., Hannon,G.J., Ruddell,C.J. and Beach,D. (1994) Construction of an improved host strain for two hybrid screening. *Nucleic Acids Res*., **22**, 1502–1503.

Fernandez-Sarabia,M.J. and Bischoff,J.R. (1993) Bcl-2 associates with the ras-related protein R-ras p23. *Nature*, **366**, 274–275.

González-García,M., Pérez-Ballestero,R., Ding,L., Duan,L., Boise,L.H., Thompson,C.B. and Núñez,G. (1994) *bcl-*x_L is the major *bcl-x* mRNA form expressed during murine development and its product localizes to mitochondria. *Development*, **120**, 3033–3042.

Han,J., Sabbatini,P., Perez,D., Rao,L., Modha,D. and White,E. (1996a) The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev*., **10**, 461–477.

Han,J., Sabbatini,P. and White,E. (1996b) Induction of apoptosis by human Nbk/Bik, a BH3-containing protein that interacts with E1B 19K. *Mol. Cell. Biol*., **16**, 5857–5864.

Hannon,G.J., Demetrick,D. and Beach,D. (1993) Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. *Genes Dev*., **7**, 2378–2391.

Hockenbery,D., Núñez,G., Milliman,C., Scheiber,R.D. and Korsmeyer,S.J. (1991) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, **348**, 334–336.

Krajewski,S., Tanaka,S., Takeyama,S., Schibler,M.J., Fenton,W. and Reed,J.C. (1993) Investigation of the subcellular distribution of the *bcl-2* oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res*., **53**, 4701–4714.

Merino,R., Grillot,D.A., Simonian,P.L., Muthukkumar,S., Fanslow,W.C., Bondada,S. and Núñez,G. (1995) Modulation of anti-IgM-induced B cell apoptosis by Bcl-X_L and CD40 in WEHI-231 cells: dissociation from cell cycle arrest and dependence of the antibody–IgM receptor interaction. *J. Immunol*., **155**, 3830–3838.

Motoyama,N. *et al*. (1995) Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*, **267**, 1506–1510.

Muchmore,S.W. *et al*. (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature*, **381**, 335–341.

Núñez,G., London,L., Hockenbery,D., Alexander,M., McKearn,J.P. and Korsmeyer,S.J. (1990) Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J. Immunol*., **144**, 3602–3610.

Oltvai,Z.N. and Korsmeyer,S.J. (1994) Checkpoints of dueling dimers foil death wishes. *Cell*, **79**, 189–192.

Oltvai,Z.N., Milliman,C.L. and Korsmeyer,S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609–619.

Sato,T. *et al*. (1994) Interactions among members of the Bcl-2 family analyzed with a yeast two-hybrid system. *Proc. Natl Acad. Sci. USA*, **91**, 9283–9242.

Shaw,G. and Kamen,R. (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*, **46**, 659–667.

Simonian,P.L., Grillot,D.A.M., Merino,R. and Núñez,G. (1996) Bax can antagonize Bcl-X_L during etoposide and cisplatin-induced cell death independently of its heterodimerization with Bcl-X_L. *J. Biol. Chem*., **271**, 22764–22772.

Swaroop,A. and Xu,J. (1993). cDNA libraries from human tissues and cell lines. *Cytogenet. Cell Genet*., **64**, 292–294.

Takayama,S., Sato,T., Krajewski,S., Kochel,K., Irie,S., Millan,J.A. and Reed,J.C. (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell*, **80**, 279–284.

Thompson,C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456–1462.

Vaux,D.L., Cory,S. and Adams,J.M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*, **335**, 440–442.

Veis,D.J., Sorenson,C.M., Shutter,J.R. and Korsmeyer,S.J. (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic disease, and hypopigmented hair. *Cell*, **75**, 229–240.

Wang,H.G., Takayama,S., Rapp,U.R. and Reed,J.C. (1996) Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc. Natl Acad. Sci. USA*, **93**, 7063–7068.

White,E. (1996) Life, death and the pursuit of apoptosis. *Genes Dev*., **10**, 1–15.

White,E., Cipriani,R., Sabbatini,P. and Denton,A. (1991) Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. *J. Virol.*, **65**, 2968–2978.

Yamagata,K., Sanders,L.K., Kaufmann,W.E., Yee,Y., Barnes,C.A., Nathans,D. and Worley,P.F. (1994) RheB, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. *J. Biol. Chem*., **269**, 16333–16339.

Yang,E., Zha,J., Jockel,J., Boise,L.H., Thompson,C.B. and Korsmeyer,S.J. (1995) Bacl, a heterodimeric partner for Bcl-xL and Bcl-2, displaces Bax and promotes cell death. *Cell*, **80**, 285–291.

Zha,H., Aimé-Sempé,C., Sato,T. and Reed,J.C. (1996) Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. *J. Biol. Chem*., **271**, 7440–7444.

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