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Human Homologue of S. *Pombe* Rad9 Interacts with Bcl-2/Bcl-x_L and Promotes Apoptosis

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ABSTRACT

DNA damage induces apoptosis through a Bcl-2 suppressible signaling pathway, but the mechanism is unknown. The human cell cycle checkpoint control protein HRad9 was found to interact with Bcl-2 and Bcl-XL but not Bax or Bad, as demonstrated by yeast two-hybrid and coimmunoprecipitation studies. When overexpressed in mammalian cells, HRad9 induced apoptosis that can be blocked by B&2 or Bcl-XL. Conversely, HRad9 antisense RNA suppressed cell death induced by methyl methanesulfonate. These findings imply that HRad9 has a novel role in potentially regulating apoptosis after DNA damage, in addition to its previously described checkpoint control and other radioresistance promoting functions.

INTRODUCTION

The B&2 family of proteins consists of both suppressors and promoters of programmed cell death and apoptosis. Many of the B&2 family proteins can interact with each other through a complex network of homo- and hetero-dimers. The relative ratios of pro- and anti-apoptotic members of this family determine whether the cell should live or die I. Sequence comparisons have identified up to four evolutionarily conserved domains within Bcl-2 family proteins, called Bcl-2 Homology (BH) domains: BHI, BH2, BH3, and BH4. Mutagenesis studies have revealed that the conserved BH3 domain within many of the pro-apoptotic Bcl-2 family members is critical for binding to anti-apoptotic proteins such as Bcl-2 and Bcl-xL, and also for their pro-apoptotic activity *. Several pro-apoptotic proteins including Bik, Bim, Bid, Hrk, Bad, and nematode Egl-1 share only the BH3 domain with other members of the Bcl-2 family, constituting a new category called the BH3-only subfamily of the Bcl-2 family 2,s. Most of these BH3-only pro-apoptotic proteins appear to function essentially as trans-dominant inhibitors by binding to anti-apoptotic Bcl-2 family members and neutralizing their cell survival activity Z3.

Cell cycle checkpoints monitor DNA replication and repair, thereby coupling the completion of these events to the onset of mitosis 4. In relatively simple organisms such as yeasts, checkpoint controls are critical for survival when DNA replication is inhibited or DNA is damaged, while in multicellular organisms they play an important role in maintenance of genomic integrity; loss

of genomic integrity can cause cancer and genetic diseases. Many of the genes required for cell cycle arrest in response to DNA damage have also been shown to be required for DNA repair, apoptosis, and transcriptional induction 5. In fission yeast, a group of six checkpoint genes, including **r&f'**, *rad3', rad9', rad77',* r&26', and *bus?'* are responsible for arresting cells in the G2-phase of the cell cycle before mitotic entry in the presence of DNA damage or incomplete DNA replication s-9. We provide evidence here that the human homologue (HRad9) of *S. pombe* Rad9 can interact with Bcl-2 and Bcl-XL through a BH3-like region located in the N-terminus of the HRad9 protein, and thus promote apoptosis in cells.

RESULTS

HRad9 Contains a BH3-Like Region. To identify novel apoptosis-regulatory proteins, the EMBL non-redundant protein sequence database was screened for proteins with homology to the Bcl-2 homology 3 (BH3) domain by using a peptide search program. The search reports revealed that the human homologue of S. *pombe* Rad9 contains a stretch of 15 amino acids near its N-terminus (amino acids 16 to 30) with conserved critical residues found in the BH3 domains of pro-apoptotic Bcl-2 family proteins (Fig. 1). It has been reported that the conserved BH3-like regions of many pro-apoptotic Bcl-2 family proteins are critical for their induction of cell death and for their interactions with anti-apoptotic proteins such as Bcl-2 and Bcl-XL 10-17.

HRad9 Binds to Bcl-2 Family Proteins. Initially, yeast two-hybrid assays were employed to test the ability of HRad9 to interact with B&2 family proteins. Co-expression of HRad9 fused with a trans-activation domain (AD-HRad9) and Bcl-2 fused with a LexA DNA-binding domain (LexA-Bcl-2) resulted in transactivation of a /acZ reporter gene under the control of LexA operators, as determined by p-galactosidase activity assays (Fig. 2A). However, LexA-Bcl-2 protein failed to form two-hybrid interactions with AD-HRadSAN, a deletion mutant of HRad9 protein lacking the first 27 amino acids and thus devoid of the BH3-like region (Fig. 2A). In addition, two-hybrid analysis of a mutant LexA-HRad9 protein that contained only the first 39 amino acids of HRad9 where the BH3-like domain resides demonstrated that this portion of the HRad9 protein is sufficient for interactions with Bcl-2 (Fig. 2A). Similar results were obtained for the LexA/Bcl-xL protein (Fig. 2A), indicating that it too is a HRad9 binding partner. In contrast, LexA-Bax and LexA-Bad proteins produced little or no transactivation of the /acZ reporter gene when co-expressed with AD-HRad9. The LexA-Bax and LexA-Bad fusion proteins did interact with Bcl-2 or B&XL, confirming successful expression of these proteins (Fig. 2A). The specificity of the association between B&2 or Bcl-xL and HRad9 was further demonstrated by the failure of these proteins to form two-hybrid interactions with v-Ras and by the absence of two-hybrid interactions between HRad9 and Bax or Bad (Fig. 2A).

Coimmunoprecipitation experiments were performed to determine whether similar interactions could occur between HRad9 and Bcl-2 or Bcl-XL in

mammalian cells. In 293 cells coexpressing Bcl-2 and Flag-tagged HRad9 or the HRadSAN mutant, Bcl-2 was coimmunoprecipitated with the wild-type but not the AN HRad9 protein (Fig. 28). Immunoprecipitations performed using anti-Myc antibody confirmed the specificity of these results. In addition, endogenous Bcl-2 can be coimmunoprecipitated with HA-HRad9 from FL5.12 cells stably expressing HA-epitope tagged HRad9 (Fig. 2C). Importantly, in the human leukemic cell line K562, endogenous HRad9 could be coimmunoprecipitated with endogenous Bcl-2 but not Bad (Fig. 2D), providing further evidence that the HRad9-Bcl-2 interaction occurs under physiological protein level conditions. Similarly, interactions between Bcl-XL and HRad9 also were detected in 293 cells expressing Bcl-xL and Flag-tagged HRad9 protein (Fig. 2E).

To explore further the interaction of Bcl-2 and HRad9, immunofluorescence analysis was performed in Hela epithelial cells. Bcl-2 was expressed as a green fluorescent protein (GFP) fusion protein with either wild-type HRad9 or the HRad9DN mutant. Fluorescence confocal microscopic analysis revealed a granular and extranuclear distribution of GFP-Bcl-2 (Fig. 3, a, d, g, and j), consistent with a localization confined to intracellular membranes including the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum 18, 1s. Cells labeled with anti-Flag monoclonal antibody showed that Flag-HRad9 protein was located throughout the cell, with more being concentrated in the nucleus and partially colocalized with GFP-Bcl-2 on the intracellular membranes (Fig. 3, *b* and c). Following treatment with methyl methanesulfonate (MMS), a DNA-damaging agent, Flag-HRad9 protein showed

most exclusively pen-nuclear immunostaining and colocalization with Bcl-2 (Fig. 3, e and f>. The peri-nuclear translocation of HRad9 protein was evident within 1 hr, preceding apoptosis that was detectable at -8 hrs after MMS treatment (not shown). Interestingly, removal of the BH3-like domain from the HRad9 protein eliminated the cytoplasmic distribution and colocalization with Bcl-2 (Fig. 3, *h* and i). These results are consistent with this mutant protein being resistant to MMS-induced peri-nuclear redistribution (Fig. 3, k and *l*), unable to cause apoptosis (Fig. 5), and incapable of coimmunoprecipitation with Bcl-2 (Fig. 2B, lanes 4-6). Taken together, these findings indicate that Bcl-2 associates with HRad9 and that the BH3-like region of HRad9 is required for these interactions.

Rad9 Promotes Apoptosis in Mammalian Cells. To study the effects of HRad9 alone or in combination with Bcl-2 on cell survival, Interleukin-3 (IL-3) dependent FL512 cells were stably cotransfected with expression plasmids encoding HA-epitope tagged HRad9 and/or Bcl-2. Immunoblot analysis of lysates prepared from these cells confirmed the expression of these transgenes (Fig. 2C). When deprived of IL-3, FL5.12 cells synthesizing the HRad9 protein died with accelerated kinetics relative to control-transfected Neo cells (Fig. 4A). In the experiment shown, for example, only -2% of FL5.12-Rad9 cells remained viable at 24 hr after growth factor withdrawal, compared to -30% of the FL5.12-Neo control cells. Coexpression of Bcl-2 essentially abrogated these effects of HRad9. The kinetics of FL5.12-Bcl-2/HRad9 cell death were comparable to those of FL5.12-Bcl-2 cells.

Next, flow cytometric analysis of propidium iodide (PI) stained cells was performed to measure the DNA content of the transfected FL5. 12 cells when cultured in the presence or absence of IL-3. When provided with exogenous IL-3, all of the FL5. 12 cell transfectants displayed the usual range of DNA contents reflecting cells in G_0/G_1, S, and G2/M-phases of the cell cycle, regardless of transfection with HRad9 or Bcl-2. As shown in Fig. 4B, for example, the relative portion of FL5.12-HRad9 cells in G0/G1, S, and G2/M-phases was 66%, 22%, and 12%, respectively, compared to ranges of 64-66% (G_0/G_1), 20-24% (S), and 13-16% (G_2/M) among FL5. 12-Bcl-2 and FL5.12-Neo cells. Thus, HRad9 did not significantly alter the cell cycle distribution of FL5.12 cells. When cultured for 12 hrs in the absence of IL-3, however, a large proportion (-41%) of FL5.12-HRad9 cells contained degraded DNA that occurs during apoptosis, compared to -10% of the FL5.12-Neo control cells. The remaining presumably viable cells with normal DNA contents were distributed in all phases of the cell cycle, with 67% in G_0/G_1, 23% in S, and 10% in G_2/M-phases. In contrast, FL5.12 cells expressing high levels of Bcl-2 retained mostly normal DNA contents >95%, regardless of whether HRad9 protein was also present at high levels in the cells. Taken together, these observations demonstrate that HRad9 does not alter the cell cycle distribution of FL5.12 cells when cultured with saturating amounts of lymphokines, but does increase the rate of apoptosis when IL-3 is lacking.

To determine whether the BH3-like region of HRad9 is necessary for induction of apoptosis, we used a transient transfection assay in 293 cells to compare the pro-apoptotic activity of wild-type HRad9 and a deletion mutant

lacking the BH3-like region. The dead cells were assessed 2 days later by DAPI staining to distinguish apoptotic cells from those that retained normal nuclear morphology. As shown in Fig. 5A, more than half of the cells transfected with plasmids producing HRad9 showed typical apoptotic nuclear morphology (Fig. 5A, *a),* which can be inhibited by expression of Bcl-x_L (Fig. 5A, b). In contrast to the wild-type HRad9 protein, the BH3-like region deletion mutant showed a dramatic reduction in the ability to induce cell death (Fig. 5A, c). To further verify these observations, we introduced GFP-HRad9 proteins into MCF7 cells with or without Bcl-2. After 2 days of lipofectamine transfection, the viability of GFP-positive cells was assessed based on the ability to exclude PI dye. As shown in Fig. 5B, only -15 of HRad9ΔN mutant-producing MCF7 cells died, compared to over 50% of those expressing intact HRad9. The cell death induced by HRad9 was dramatically reduced by coexpression of Bcl-2.

HRad9 Antisense RNA Blocks MMS-Induced Cell Death. A human *HRad9* cDNA was subcloned into the pcl-Neo vector in reverse orientation and transiently transfected into Hela cells with Flag-HRad9-producing plasmids to verify antisense-mediated down-regulation of HRad9 protein. As shown in Figure 6A, the HRad9 antisense (AS) plasmid markedly reduced the levels of Flag-HRad9 protein compared to control transfected cells. In contrast, the HRad9-AS plasmid had no effect on the levels of Bax or other proteins examined, confirming the specificity of the results. To determine whether the antisense-reduced levels of HRad9 protein correlate with alterations in sensitivity to DNA damage-induced

cell death, the HRad9-AS plasmid was transiently transfected into Hela cells with a *lac*Z reporter plasmid. The HRad9-AS plasmid protected Hela cells from MMS-induced cell death in a concentration-dependent manner (Fig. 6B), as determined by X-gal staining. As representative samples show in Figure 6C, X-gal positive (blue) Hela cells that received the HRad9-AS plasmid were flat and well attached to the dish, whereas control transfected cells were round, indicative of a typical morphology of dead cells. These antisense RNA experiments provide further evidence that HRad9 regulates programmed cell death in human cells.

DISCUSSION

The observations described in this report suggest a novel role for HRad9 as an inducer of apoptotic cell death, in addition to its previously described cell cycle checkpoint control and other radioresistance promoting functions [6, 8, 20, 21]. We found that the human G2 checkpoint control protein HRad9 contains a region bearing similarity to the BH3 domain but lacks the conserved BH1, BH2, and BH4 amino acid sequences. Deletion experiments indicated that the BH3-like region of the HRad9 protein is critical for the cell death inducing function and also for interactions with the anti-apoptotic proteins Bcl-2 and Bcl-x_L, consistent with properties of other BH3-containing pro-apoptotic proteins [10-17]. Interestingly, HRad9 appears as three distinct species on western blots, representing alternative, extensively modified versions of the protein [22, 23]. Indeed, treatment of the HRad9-containing cell lysates with alkaline phosphatase removed the

slower-migrating bands (not shown) [22, 23], suggesting that HRad9 is phosphorylated. HRad9 has been shown to associate with hRad1 and hHus1, but phosphorylation is not an absolute requirement for this complex formation [22, 23], implying that phosphorylation might regulate HRad9 interactions with other members of the DNA damage checkpoint-signaling cascade. It is worth noting that two forms of Flag-HRad9 were detected from the immune complexes prepared using antibodies specific for either Bcl-2 (Fig. 2B; lane 3) or Bcl-x_L (Fig. 2E; lane 3), raising the possibility that this modification is required for association of HRad9 with Bcl-2 or Bcl-x_L. In addition, deletion of the BH3-like region from the HRad9 protein reduced the extent of the post-translational modifications, abrogated its ability to interact with Bcl-2 (Fig. 2B), and eliminated its apoptosis-inducing activity (Fig. 5). However, we can not exclude the possibility that removal of the BH3-like region causes a conformational change of the HRad9 molecule.

The *S. pombe rad9* gene was originally isolated from a genomic DNA library and was capable of reducing the radiosensitivity of *rad9-192* mutant cells [24, 25]. The inability to delay cell cycle progression after radiation exposure accounts for at least part of the radiosensitivity of *rad9* mutant cells, though the lack of other cell cycle independent functions of Rad9 may also contribute [26]. The connection between radiosensitivity and cell death is not clear in *S. pombe.* We compared the sensitivity of *rad*9$^+$ and *rad*9-null *S. pombe* cells to cytotoxic agents. The current (not shown) and previously published results [20, 25] indicate that *rad*9 mutant cells containing *rad*9::*ura*4 or an alteration in the BH3-like

region are more sensitive to UV, gamma-rays, or MMS treatment compared to the *rad9*$^{+}$ strain, as demonstrated by colony formation assays and phloxin B staining. These findings strongly argue that the cell death pathways in fission yeast differ from those found in mammals since classically the loss of a pro-apoptotic protein would predictably make cells more resistant, not more sensitive, to these agents. Indeed, no Bcl-2-like anti-apoptotic proteins have been found in yeast and no caspase homologues exist in **S.** pombe. Nevertheless, our results obtained from mammalian cells strongly suggest that HRad9 functions much like p53 or ATM, which controls multiple downstream pathways including cell cycle checkpoint control and apoptosis [27-29]. Other investigations indicate that there is the lack of an identifiable p53 gene in yeast, again arguing for a different apoptotic-like mechanism in S. *pombe.* The molecular mechanism by which Rad9 commits the cell to transiently delay cell cycle progression or die after DNA damage is not known, however, our findings open up several avenues of investigation that will presumably form the basis of future related studies.

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METHODS

Plasmids: *S. pombe rad9* and human *Hrad9* cDNAs [20, 25] were employed as templates for polymerase chain reactions (PCR) to amplify DNA. Mutations were created using a two-step PCR method [30]. The *rad9* DNA fragments with engineered EcoRI and XhoI sites were cloned in frame into the EcoRI/XhoI-digested HA-tag vector pcDNA3-HA [31], the pEGFP-C2 (Clontech Laboratories Inc.) vector, and the yeast two-hybrid vectors pGilda and pJG4-5 [32], or the EcoRI/SalI-digested FLAG-tag vector pFLAG-CMV2 [32]. The internal EcoRI site in the ORF region of human *Hrad9* was destroyed by PCR mutagenesis. All other plasmids have been described [31-33].

Immunoprecipitation and Immunoblot Assays: For co-immunoprecipitation experiments, cells were lysed in 0.6 ml of NP-40 lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH8.0, 1.5 mM MgC12, 1 mM EDTA, 0.2% NP-40) containing 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 5 mM NaF, 1 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, and 10 mM sodium+-glycerophosphate. Immunoprecipitations were performed by incubating 0.2 ml of lysates with 20 μl of protein G-Sepharose preadsorbed with 5 μg of anti-FLAG M2 antibody (Kodak, Inc), anti-HA 12CA5 antibody (Boehringer Mannheim), or anti-Myc 9E10 as a negative control for 3 hr at 4°C. The beads were washed 3x with 1 ml NP-40 lysis buffer supplemented with protease and phosphatase inhibitors, boiled in Laemmli buffer, and the eluted proteins were subjected to 12% SDS-PAGE/immunoblot

analysis using rabbit antisera [32] specific for Bcl-x_L, Bcl-2, SpRad9, or HRad9 and ECL-based detection (Amersham).

Yeast Two-Hybrid Assays: S. *cerevisiae* EGY48 cells were transformed with various combinations of the pGilda expression plasmids producing LexA DNA-binding domain fusion proteins and pJG4-5 plasmids encoding B42 transactivation domain fusion proteins [32, 34]. Three independent transformants were grown in either galactose or glucose containing media, to either induce or repress, respectively, the *gal-1* promoters in the two-hybrid plasmids. For these assays, the transmembrane domains of Bcl-2 and Bax were deleted to avoid problems with nuclear import. Removal of the membrane-anchoring region from Bax also abolishes its cytotoxicity in yeast [35].

Immunofluorescence Analysis: Cells were fixed in 4% paraformaldehyde/PBS (pH7.4) for 1 hr at 4 °C and permeabilized in 0.5% Triton X-100 for 1 hr. After preblocking in 5% (v:v) skim milk and 3% (v:v) BSA for 1 hr, cells were incubated with anti-FLAG M2 monoclonal antibody (50 mg/ml; SIGMA) which was detected with rhodamine-conjugated rabbit anti-mouse IgG (1:20; DAKO). Immunofluorescent staining was analyzed using a confocal laser-scanning microscope (Model GB-200, OLYMPUS).

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Figure 1. Alignment of amino acid residues in the BH3 homology regions of Bcl-2 protein family members with HRad9. Comparison of the BH3-like regions in HRad9 and Bcl-2 family members (human Rad9, accession number U53174; human Bax, L22474; human Bak, U23765; mouse Bid, U75506; human Bik, U34584; human Hrk, U76376; human Bad, AF021792; human Bim, AF032457; C. *elegans* Egl-1, AF057309). Amino acids that match the BH3 consensus (PS01 259) are darkly shaded.

Figure 2. HRad9 selectively interacts with Bcl-2 family proteins. (A) Yeast two-hybrid assays. Interactions of pairs of fusion proteins containing either a N-terminal LexA DNA-binding domain (BD) or a B42 transactivation domain (AD) were tested by the yeast two-hybrid assay using *LEU2* and *lacZ* reporter genes under the control of *lexA* operators. Data were scored as positive (+) or negative (-) for interaction. In all (+) cases, unambiguous growth and production of an intense blue color on a leucine-deficient agar plate containing X-gal were obtained from three independent transformants. Negative (-) interactions yielded either no or very little growth on leucine-deficient medium or blue color for the β-galactosidase assay. Data are representative of at least three independent experiments.

(B) Coimmunoprecipitation assays. 293 cells were transiently transfected with 5 μg of pRC/CMV-Bcl-2 and 10 μg of pFlag-CMV2 plasmids encoding human Rad9 or Rad9ΔN, which lacked the first 27 amino acids of the wild-type protein.

Cell lysates were prepared 2 days later and immunoprecipitation was performed using monoclonal antibodies specific for Flag, c-Myc, or Bcl-2, followed by immunoblot analysis of the resulting immune-complex using either anti-HRad9 (top) or anti-Bcl-2 antisera (bottom) as a probe.

(C) Coimmunoprecipitation of endogenous Bcl-2 and HA-HRad9 from FL5.12 cells. FL5.1 2 cells were transfected with 10 μg of ScaI-linearized pcDNA3-HA-HRad9 or parental pcDNA3 vector DNA using electroporation as described [32]. Stable transfectants were selected in 1 mg/ml G418. Cell lysates were prepared and immunoprecipitation were performed using anti-HA monoclonal antibody, followed by Western blot analysis using polyclonal rabbit antisera specific for HRad9 (top) or Bcl-2 (bottom).

(D) Interactions of endogenous Bcl-2 with endogenous HRad9 in K562 cells. Lysates from cultured K562 human leukemic cells were used for immunoprecipitation with anti-Bcl-2 or anti-Bad monoclonal antibodies followed by SDS-PAGE and immunoblot analysis of the resulting immune-complexes with either anti-HRad9 (top) or anti-Bcl-2 (bottom) polyclonal antisera.

(E) Co-immunoprecipitation of Flag-HRad9 and HA-Bcl-x_L from 293 cells. 293 cells were transiently cotransfected with 10 μg of pFlag-CMV2 encoding human Rad9 and 5 μg of pcDNA3-HA-Bcl-x_L expression plasmids. Immunoprecipitation were performed 2 days later using either anti-Myc antibody as a negative control

or monoclonal antibodies specific for the Bcl-x_L or Flag-epitope. Western blots were developed with anti-HRad9 (top) or anti-Bcl-x_L (bottom) polyclonal antisera.

Figure 3. DNA-damaging agent induces peri-nuclear assembly of HRad9 and colocalization with Bcl-2. Hela cells were transiently transfected with pEGFP-Bcl-2 and pFlag-CMV2 plasmids encoding Flag-HRad9 *(a-f)* or Flag-HRad9ΔN (g-l). After 1 day, the cells were cultured without (a-c, g-i) or with 1 mM MMS (d-f, *j-l*) for 3 hrs, when most cells remained viable. Cells were fixed and stained with anti-Flag antibody, followed by Rhodamine-conjugated secondary antibody, and analyzed by fluorescence confocal microscopy using appropriate filters for visualization of green *(a, d*, g, and *j*), red (b, e, *h*, and k), or combined (c, *f*, i, and *l*) fluorescence resulting from the presence of GFP and Rhodamine molecules.

Figure 4. Effects of HRad9 on survival of FL5.12 cells. (A) FL5.12 cells were stably transfected with pcDNA3 (Neo), pcDNA3-HA encoding human Rad9 with or without Bcl-2, or pRC/CMV-Bcl-2 plasmids as indicated. Cells were cultured without IL-3 for various times and the percentage of viable cells was determined by trypan blue dye exclusion (mean ± SD; n=3).

(B) The relative DNA content of transfected FL5.12 cells was determined for cells cultured with (+) or without (-) 10% WEHI-3B conditioned medium for 12 hrs. Cells were fixed, permeabilized, and treated with RNAse before incubation in 50

μg/ml propidium iodide (PI) and analysis by flow cytometry as described previously [36]. The data displayed fluorescence intensity (abscissa) versus relative cell number (ordinate). DNA contents showing apoptotic cells (A_0) and viable cells in G_0/G_1, S, or G_2/M-phases of the cell cycle are indicated.

Figure 5. HRad9 promotes apoptosis in a BH3-dependent manner. (A) 293 cells were transiently transfected with a total of 3 μg DNA, including 1 μg of pFlag-CMV2 encoding HRad9, or HRad9ΔN and 2 μg of pcDNA3-Bcl-x_L or parental pcDNA3 plasmid DNA. After 2 days, the cells were fixed in 3.7% formaldehyde, stained with 0.1 μg/ml DAPI, and analyzed by fluorescence microscopy with UV filter. Data are representative of three independent experiments.

(B) MCF7 cells were transfected with 1 μg of plasmids encoding GFP-HRad9, GFP-HRad9ΔN, or control GFP and 2 μg of pRC/CMV-Bcl-2 or parental plasmid DNA. After 2 days following lipofectamine transfection, the percentage of GFP-positive dead cells was determined by PI dye staining (mean ± SD; n=3).

Figure 6. HRad9 antisense RNA suppresses cell death induced by MMS. (A) Hela cells were cotransfected with 0.6 μg of Flag-HRad9-producing plasmid in combination with either 1.8 μg of pcl-Neo or pcl-Neo-HRad9-AS. Cell lysates were prepared 2 days later, normalized for protein content (50 μg of total protein

per lane), and subjected to SDS-PAGE/immunoblot analysis using either anti-Flag (top) or anti-Bax (bottom) antibodies.

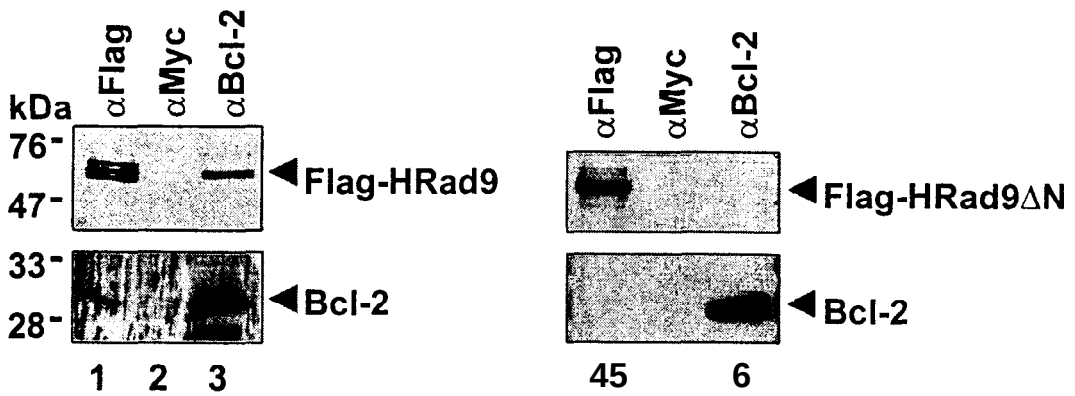
(B-C) Hela cells in 6-well plate were cotransfected with the indicated amount of pcI-Neo-HRad9-AS plasmid DNA plus 0.1 mg of pCMV-β-gal plasmid. The total amount of plasmid DNA was normalized to 0.9 μg for each transfection by adding control vector pcI-Neo. 2 days after lipofectamine transfection, cells were exposed to 1 mM MMS for 8 hrs, fixed with 2% formaldehyde, and stained in X-gal reaction mixture (1 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS) at 37 °C for 3-5 hrs. The percentage of dead cells was determined as the ratio of round blue cells among total number of blue cells counted (B). X-Gal stained cells were photographed under a light microscope (C). The data are representative of at least three independent experiments.

														D	L			
	L												L	E	V			
	I						K		L			D	I	N	S			
	V						Q		A			E	M	S	R	R		
	A						A		V			S	F	H	H	N		
	T	X	X	X	L	R	X	I		G	D	G	V	Q	Q	S		BH3 Consensus
16	A	V	H	S	L	S	R	I		G	D	E	L	Y	L	E	30	HRad9
59	L	S	E	C	L	K	R	I		G	D	E	L	D	S	N	73	Bax
74	V	G	R	Q	L	A	I	I		G	D	D	I	N	R	R	88	Bak
86	I	A	R	H	L	A	Q	I		G	D	E	M	D	H	N	100	Bid
57	L	A	L	R	L	A	C	I		G	D	E	M	D	V	S	71	Bik
33	T	A	A	R	L	K	A	L		G	D	E	L	H	Q	R	47	Hrk
110	Y	G	R	E	L	R	R	M	S	D	E	F	V	D	S		124	Bad
148	I	A	Q	E	L	R	R	I		G	D	E	F	N	A	Y	162	Bim
54	I	G	S	K	L	A	A	M	C	D	D	F	D	A	Q		68	Egl-1

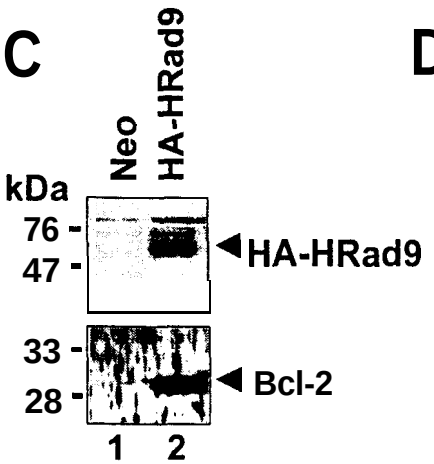
A

LexA (AD)		B42 (AD)	Gal/Leu(-)	Glu/Leu(+)	Results
Bcl-2	X	HRad9			+
Bcl-2	X	v-Ras			-
Bcl-2	X	HRad9ΔN			
Bcl-x_L	X	HRad9			+
Bcl-x,	X	v-Ras			-
Bax	X	HRad9			-
Bax	X	Bcl-2			+
Bad	X	HRad9			-
Bad	X	Bcl-x,			+
HRad9(1-39)	X	Bcl-2			+
HRad9(1-39)	X	empty			-

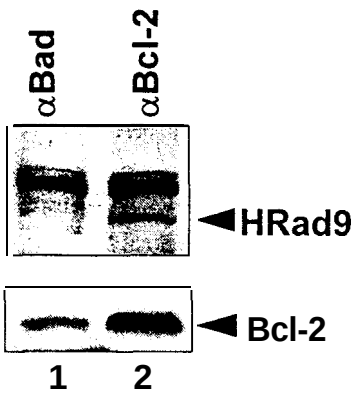
B



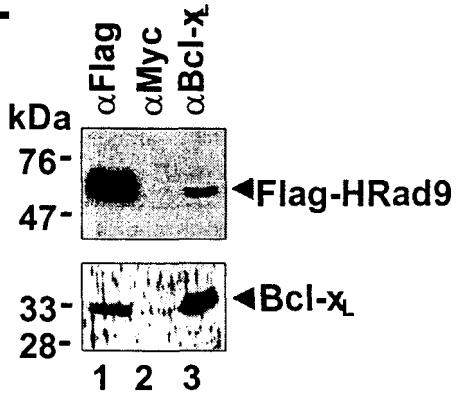
C



D

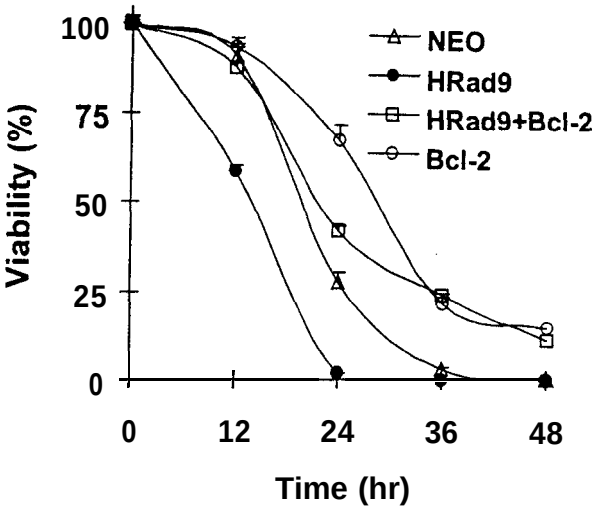


E

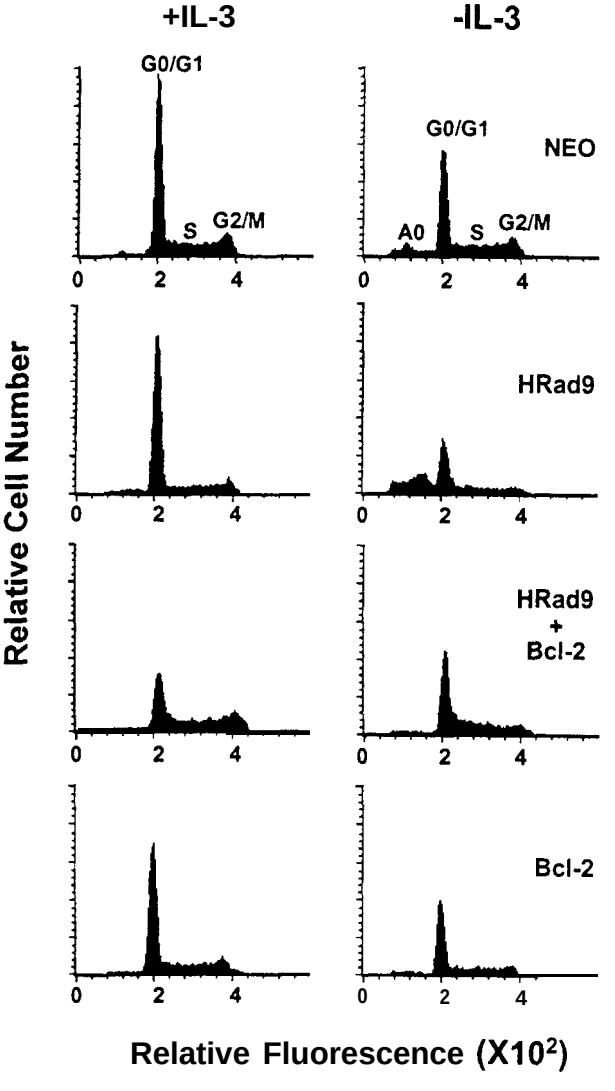


GFP
Anti-Flag
Combined
GFP-Bcl-2 + FLAG-HRad9
MMS(-)
MMS(+)
GFP-Bcl-2 + FLAG-HRad9ΔN
MMS(-)
MMS(+)
a
b
c
d
e
f
g
h
i
j
k
l

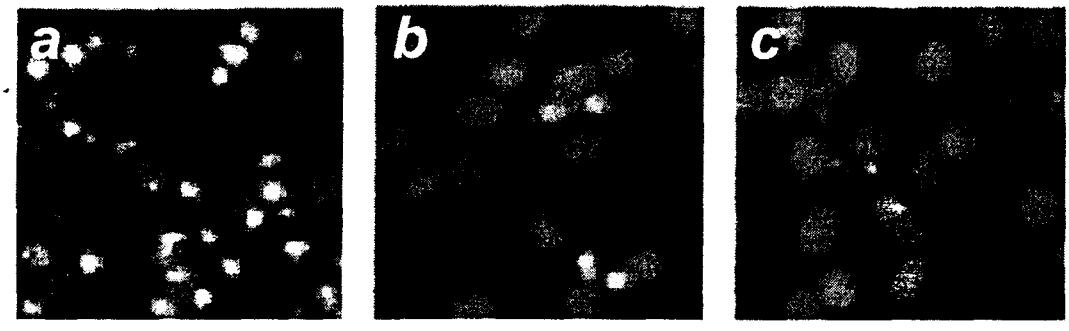
A


100
75
50
25
0
Viability (%)
0
12
24
36
48
Time (hr)
NEO
HRad9
HRad9+Bcl-2
Bcl-2

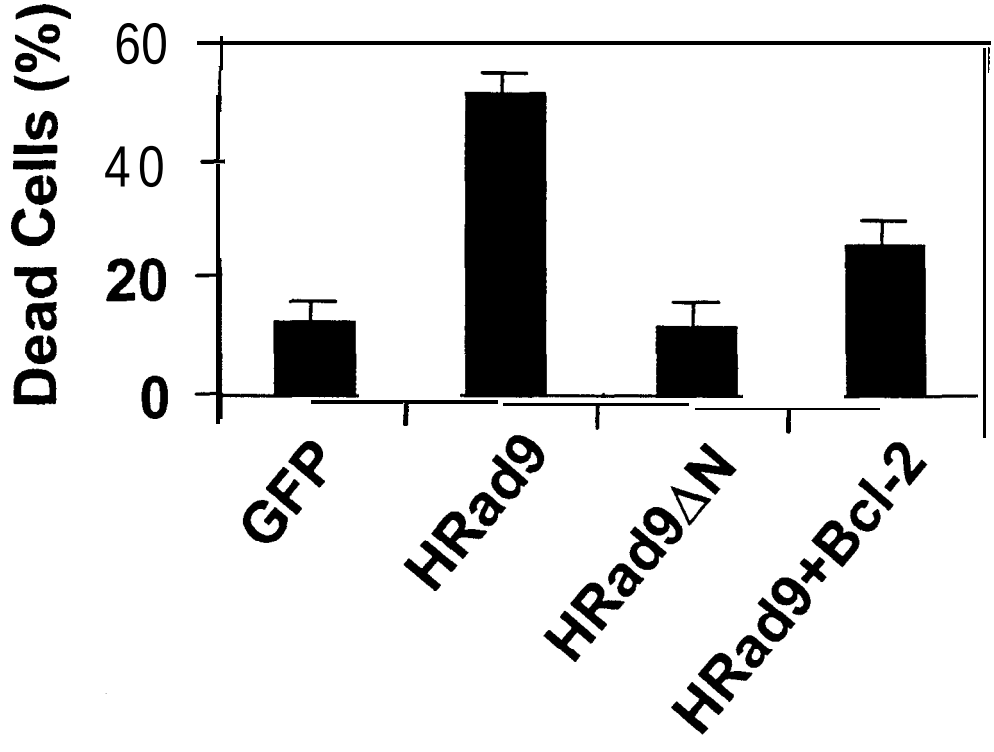
B

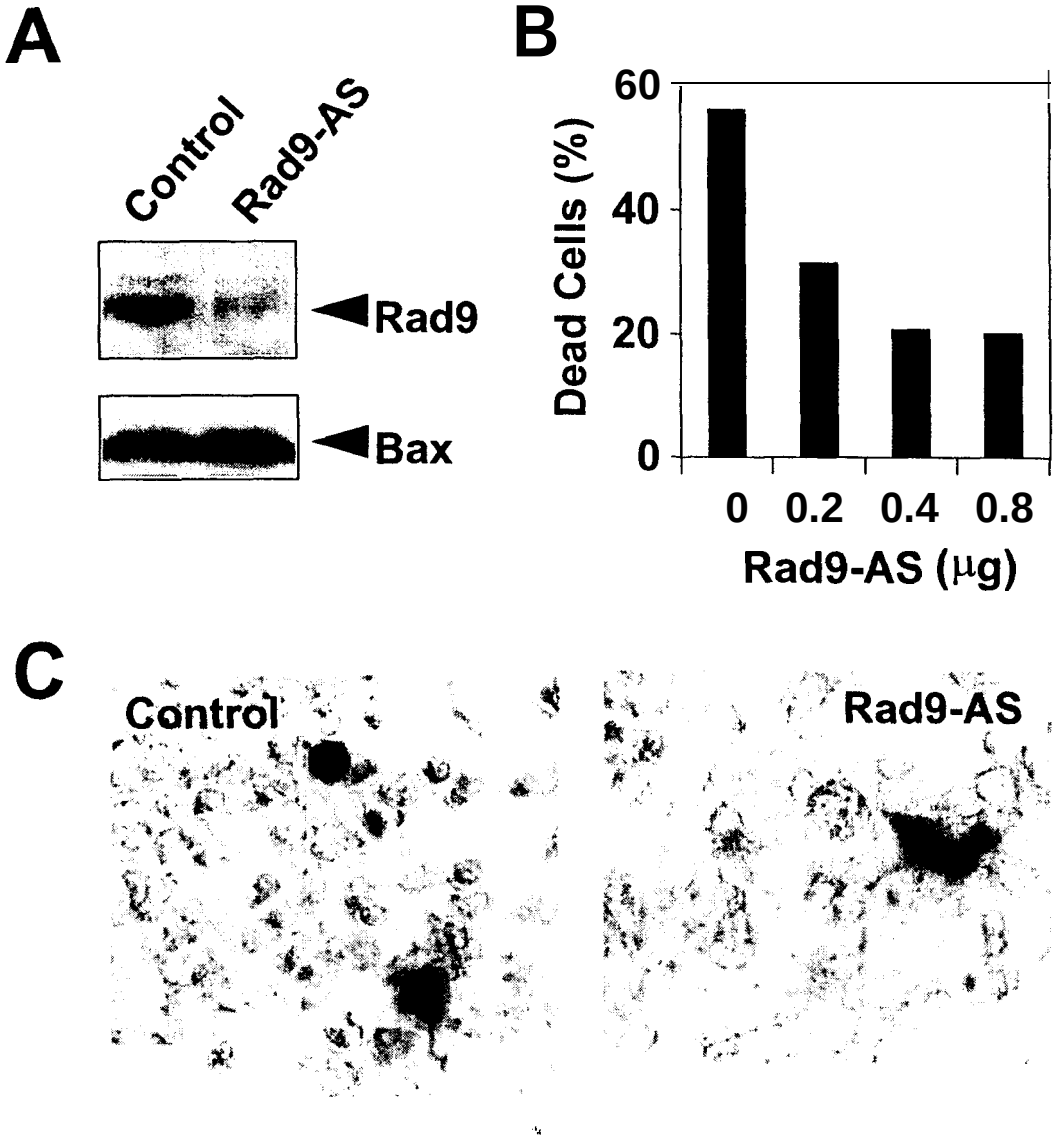

+IL-3
-IL-3
G0/G1
S
G2/M
A0
NEO
HRad9
HRad9
+
Bcl-2
Bcl-2
Relative Cell Number
0
2
4
Relative Fluorescence (X10²)

A


HRad9
HRad9 + Bcl-x$_L$
HRad9ΔN
a
b
c

B


Dead Cells (%)
60
40
20
0
GFP
HRad9
HRad9ΔN
HRad9+Bcl-2


A
Control
Rad9-AS
Rad9
Bax
B
Dead Cells (%)
60
40
20
0
0
0.2
0.4
0.8
Rad9-AS (μg)
C
Control
Rad9-AS