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HIP1R Interacts with a Member of Bcl-2 Family, BCL2L10, and Induces BAK-dependent Cell Death

Jae-Hong Kim[a], Seongmin Yoon[a], Miae Won[a], Se-Hoon Sim[b], Jeong-Jae Ko[a], Seung Hyun Han[c], Kyung-Ah Lee[a], Kangseok Lee[b] and Jeehyeon Bae[a]

[a]Graduate School of Life Science and Biotechnology, Pochon CHA University School of Medicine, Seongnam, Korea; [b]Department of Life Science, Chung-Ang University, Seoul, [c]Department of Oral Microbiology and Immunology, School of Dentistry, Seoul National University, Seoul

Key Words

BCL2L10 • Diva • BCL-B • HIP1R • BAK • Apoptosis • Caspase • Mitochondrial membrane potential

Abstract

The Bcl-2 family members are evolutionarily conserved and crucial regulators of apoptosis. BCL2L10 (human Diva or BCL-B) is a member of the Bcl-2 family that has contradictory functions in apoptosis. In the present study, we identified the Huntington-interacting protein 1-related (HIP1R) protein following a search for Diva-interacting proteins using the yeast two-hybrid system. HIP1R is a multi-domain protein that regulates the clathrin-mediated endocytic machinery and actin assembly in cells. Interaction of endogenous proteins of BCL2L10 and HIP1R in 293T cells was determined by immunoprecipitation, and their direct association was confirmed by the Far-Western analysis. The deletion of both the AP180-homology (ANTH) and F-actin-binding the talin-HIP1/R/Sla2p actin-tethering C-terminal homology (THATCH) domains of HIP1R greatly compromised its binding ability to BCL2L10. Ectopic expression of HIP1R resulted in moderate cell death of 293T cells in conjunction with the dissipation of mitochondrial membrane potential and caspase 9 activation. A member of proapoptotic Bcl-2 family, BAK, was required for HIP1R to induce cell death, while BAX was dispensable. In addition, BCL2L10 was associated with endogenous caspase 9, and their binding was augmented by HIP1R overexpression. Thus, this study provided the previously unknown function of HIP1R involved in the intrinsic cell death pathway and further explored possible mechanisms by which HIP1R induces cell death.

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Introduction

Apoptosis is essential in maintaining the homeostasis of normal cells, and its dysregulation leads a wide variety of disorders including cancer and neurodegenerative diseases [1]. Evolutionarily conserved members of the Bcl-2 family proteins are central regulators of apoptosis in diverse species [2, 3]. Mouse Diva (Boo) was initially cloned by two separate groups who reported contradictory observations; one group reported Diva was pro-apoptotic [4], while the other described it as an anti-apoptotic

member of the Bcl-2 family [5]. Diva forms a ternary complex with apoptosis-activating factor-1 (Apaf-1) and caspase-9, components of the apoptosome along with cytochrome c [5, 6]. The closest human homolog of Diva was later identified and designated human Boo/Diva, BCL2L10, or BCL-B [7-9]. BCL2L10 is also known to function as either a pro- or anti-apoptotic factor depending on the cellular context [7, 8].

In the search of Diva-interacting proteins, we identified Huntington-interacting protein 1-related (HIP1R), which was originally discovered by its sequence homology with HIP1 [10]. HIP1R protein consisted of the AP180-homology (ANTH) domain mediating phosphoinositides-interaction [11], a coiled-coil domain containing leucine zipper, and an F-actin-binding the talin-HIP1/R/Sla2p actin-tethering C-terminal homology (THATCH) domain [12, 13]. The yeast homologue of HIP1R, Sla2p, is required for endocytosis and a functional actin cytoskeleton [14-16]. Accordingly, the well-characterized functions of HIP1R in mammalian cells involve clathrin-mediated endocytic machinery and actin assembly [17, 18].

HIP1 and HIP1R share both structural and functional homologies [10, 19-21]. While HIP1-induced cell death has been demonstrated [22-25], the role of HIP1R in the apoptotic signaling pathway has not been reported in detail. In the present study, we described HIP1R as a specific binding partner of BCL2L10, tested for the functional effects of HIP1R in apoptosis, and provided evidence for the involvement of HIP1R in the intrinsic cellular death pathway.

Materials and Methods

Chemicals and reagents
Unless otherwise indicated all the chemicals were purchased from Sigma (St Louis, MO).

Plasmids construction
The full-length human BCL2L10 was cloned as in Kang et al. [26]. The plasmid encoding human HIP1R was kindly provided by Dr. Theodora S. Ross (The University of Michigan), and pCMV HA or Myc (Clontech, Mountain View, CA) HIP1R was subcloned after PCR amplification using the following primers: HIP1R-F (5'-CTA GAA TTC AAA TGA ACA GCA TCA AGA AC) and HIP1R-R (5'-CTA CTC GAG CTA GTA GTT CAC GAG TTG AGC). The ΔANTH HIP1R insert DNA was PCR amplified with HIP1R-F ΔANTH (5'-CTA GAA TTC AAA TGC AGT TTC CCG CGG GCC TG) and HIP1R-R. The ΔTHATCH HIP1R mutant was amplified by PCR using the primers HIP1R-F and HIP1R-R ΔTHATCH (5'-CTA CTC GAG CTA CTT CAC CCC CGA GCT GGC). The ΔANTHΔTHATCH HIP1R mutant

was amplified by PCR using the primers HIP1R-F ΔANTH and HIP1R-R ΔTHATCH. The Δcoiled-coil HIP1R construct was produced by a recombinant PCR technique using the following primers; HIP1R-F and HIP1R-340R (5'-GCC CCT GCT CCT GAG AGA GGT CAG CCA CCA CCA), HIP1R-610F (5'-TGG CTG ACC TCT CTC AGG AGC AGG GGC) and HIP1R-R.

The pET22b+ (Novagen, San Diego, CA) HIP1R construct was produced after PCR amplification using the following primers: pET22b(+)HIP1R-F (5'-CTA CTA CAT ATG TAC CCA TAC GAT GTT CCA) and pET22b(+) HIP1R-R (5'-TAG AAG CTT GTA GTT CAC GAG TTG). To construct pGBT9 (Clontech) BCL2L10, PCR was performed with primers of 5'-ACG GAA TTC ATG GTT GAC CAG TTG CGG and 5'-CTA GGA TCC TCA TAA TAA TCG TGT CCA. The pACT2 (Clontech) HIP1R was cloned after PCR amplification using the HIP1R-F and HIP1R-R primers.

Yeast two-hybrid system
The open reading flame of Diva was fused in frame with the GAL4-binding domain (BD) in the pGBT9 yeast shuttle vector. To identify Diva-interacting proteins, yeast screening of GAL4-activation domain (AD)-tagged rat ovarian fusion cDNA library was performed as previously described [26].

Mammalian cell culture
293T and mouse embryo fibroblasts (MEFs) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Seoul, Korea) containing 10% heat-inactivated Fetal Bovine Serum (FBS) (Welgene) and 1% penicillin-streptomycin (Welgene). Wild-type, bax$^{-/-}$, bak$^{-/-}$, and bax$^{-/-}$bak$^{-/-}$ MEF cells were generous gifts from Dr. CB Thompson (University of Pennsylvania, PA, USA).

Immunoprecipitation and Western blot analysis
293T cells (3.0 x 10^6) were transfected with a total of 6 μg of the respective plasmid DNA using Welfect-EX™PLUS (Welgene) in 100 mm dishes. Cell lysates were prepared with NP-40 lysis buffer (50 mM Tris-HCl at pH 8.0, 0.15 M NaCl, and 1% NP-40) containing 10% protease inhibitor cocktail (Sigma) The lysates were centrifuged and the supernatants were precleared with normal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and Protein-G-Agarose (Upstate, Charlottesville, VA) for 2 h at 4°C. The precleared lysates were incubated with primary antibodies for 12 h followed by incubation with Protein-G-Agarose for an additional 1 h at 4°C. The immune complexes were centrifuged and washed 3 times with NP-40 lysis buffer. For quantitative protein analysis, a standard curve was established with the standard BSA solution (Pierce, Rockford, IL), and equal amounts of total protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring the proteins to membranes, the membranes were blocked with 5% nonfat dry milk. HA- or Myc-tagged HIP1R and its mutant proteins were detected with an anti-HA or anti-Myc monoclonal antibodies (Sigma), and Flag-tagged BCL2L10 was detected with rabbit anti-Flag antibodies (Sigma: F1804). For the detections of endogenous BCL2L10 and HIP1R proteins, anti-BCL2L10 (Santa Cruz Biotechnology: sc-8739) or anti-HIP1R polyclonal antibodies (BD Biosciences,

Franklin Lakes, NJ) were used, respectively. As loading controls, Western blot analyses of GAPDH and β-actin were performed with anti-GAPDH (AB Frontier, Seoul, Korea) or anti-β-actin (Santa Cruz Biotechnology: sc-47778), respectively. Membranes were stripped using stripping buffer (100 mM mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl at pH 6.8) by incubation at 50°C for 30 min and were washed with phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 at PH 7.4) containing 0.01% Tween 20 (Sigma). The secondary horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology) were incubated with the membranes at room temperature for 1 h before visualization using enhanced chemiluminescence (AB Frontier). Proteins were detected using the LAS image program (Fuji, New York, NY).

Expression and purification of recombinant HIP1R protein

The recombinant plasmid, pET22b+ HIP1R, was transformed into E. coli BL21 (DE3). The bacterial culture was induced with 100 mM IPTG (isopropyl b-D-thiogalactopyranoside) at 37°C for 2 h and harvested. The pellets were re-suspended and lysed with a French presser. The recombinant HIP1R protein was purified using Ni-NTA resin (Qiagen, Valencia, CA) as recommended by the manufacturer.

Far-Western blot analysis

The BCl2L10 protein overexpressed in 293T cells, as described in a previous section, was separated by SDS-PAGE and transferred to nitrocellulose membrane. The blot was placed in TEN 50 buffer (10 mM Tris-Hcl pH 8.0, 1 mM EDTA, 50 mM NaCl) [27] and stored at 4°C for 5 days to allow in situ renaturation of the protein. Then, the membrane was blocked with HHB (HEPES-hybridization buffer; 20 mM HEPES pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 0.05% Triton X-100) containing 5% skim milk for 1 h and incubated with HIP1R recombinant protein or BSA (bovine serum albumin) along with RNase A (0.2 μg/ml) at 4°C for overnight in HHB supplemented with 1% skim milk. The membrane was washed three times for 10 min each with HHB containing 1% skim milk and subjected to immunoblot analysis.

Determination of caspase 9 activation

293T cells (1.0 x10⁶) were plated onto 60 mm dishes and transfected with a total of 3 μg of the respective plasmid DNAs. After 24 h after transfection, cell lysates were prepared, subjected to electophorasis, and immunoblotted with anti-caspase 9 antibody (Cell signaling, Danvers, MA: #9502) that allows to detect both pro and activated forms of caspase 9. The same membrane was stripped and reprobed sequentially with anti-Flag, -Myc, -HIP1R, -BCL2L10, BAK (Santa Cruz Biotechnology: sc-832) and -β-actin antibodies. Some cells were treated with 10 μM of Z-VAD-fmk (Calbiochem, San Diego, CA) at 6 h post-transfection.

Measurement of changes in mitochondrial membrane potential (MMP)

Mitochondrial integrity was determined by assaying the loss of the mitochondrial membrane potential (Δψm) using MitoProbe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide) Assay Kit for flow cytometry (Invitrogen), as suggested by the manufacturor. Briefly, 293T cells (1.0 x 10⁶) were plated on 60 mm dishes and transfected with a total of 3 μg of the respective plasmid DNA. Cells were harvested with trysin-EDTA (Welgene) after 24 h of incubation, and the samples were then incubated with 2 μM of JC-1 for 15 min at 37°C with 5% CO2. After washing the cells with PBS, flow cytometry analysis via FACS Caliber (BD Biosciences, Franklin Lakes, NJ) was perfromed.

Cell viability assay

293T cells were seeded at a density of 3.5 x 10⁴ per well in 48-well plates. Twenty-four hours later, cells were transfected with an equal amount of total DNA (100 ng), along with 10 ng of pEGFP-N1 (Clontech) for detection of transfected cells. To determine the involvement of caspases in HIP1R-induced cell death, the transfected cells were incubated with increasing concentrations of z-VAD-fmk for 24 h. MEF cells (2 x 10⁵) were washed with fresh PBS and suspended in 10 μl of resuspension R buffer (Digital Bio Technology, Seoul, Korea) containing a total of 500 ng of pcDNA3 and 50 ng of pEGFP-N1 to allow detection of transfected cells. The cells were electroporated using a 10 μl volume-sized tip on a MicroPorator MP-100 (Digital Bio Technology) and incubated on 24-well plates containing fresh media. At 24 h post-transfection, cells were harvested with trypsin-EDTA (Welgene) and resuspended following centrifugation. GFP-positive cells were counted under the fluorescence microscope after adding Trypan blue (Sigma). Data were expressed as the percentage of viable cells when compared to the control group that received an empty control vector.

DNA fragmentation assay

293T cells (1.0 x10⁶) were plated onto 60 mm dishes and transfected with a total of 3 μg of the respective plasmid DNAs. The cells were harvested after 24 h of incubation by lysis buffer (0.1% Triton X-100, 5 mM Tris-HCl at pH 8.0, and 20 mM EDTA) and centrifuged at 12,000 x g for 15 min at 4°C. The supernatant fraction containing fragmented DNA was mixed with 0.5 ml of isopropanol at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4°C. The DNA pellet was washed with 75% ethanol, resuspended in Tris-HCI (pH 8.0) containing 100 μg/ml of RNAse, and incubated for 2 h. The 5 μg of DNA fragments was separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light.

Statistical Analysis

Significance of values when compared to the controls was determined by t-test.

Fig. 1. BCL2L10 directly interacts with HIP1R *in vivo*. (A) Yeast cells were cultured in selective media lacking tryptophan, leucine, histidine, and adenine. Growth was demonstrated in colonies expressing both HIIP1R and BCL2L10 fused to the GAL4 DNA activation domain and binding domain, respectively. No growth of yeast was seen in colonies expressing only HIIP1R or BCL2L10. (B) 293T cells (3.0 x 10^6) were transfected with 3 μg of pcDNA3 Flag-tagged BCL2L10, Myc-tagged HIP1R, or both and incubated for 24 h. As a control, cells were transfected with the pcDNA3 empty vector (6 μg). The pre-cleared cell lysates were used for immunoprecipitation (IP) experiments with the anti-Myc or anti-Flag antibodies. Following IP, immunoblotting (IB) were performed and the immunoprecipitated proteins were detected with anti-Flag or anti-Myc antibodies. (C) To identify the endogenous interaction between BCL2L10 and HIP1R, 293T cell lysates were immunoprecipitated with the anti-HIP1R antibody, anti-BCL2L10 antibody, or normal IgG followed by Western blots with anti-BCL2L10 or anti-HIP1R antibodies. (D) Far-Western blot analysis was conducted with the BCL2L10-overexpressing mammalian cell lysate and the purified recombinant HIP1R protein. Recombinant HA-HIP1R purified using Ni-NTA resin was analyzed on a SDS-PAGE and detected with the HA antibody (left panel). 293T cells (1.0 x 10^6) were transfected with 3 μg of pcDNA3 empty vector or pcDNA3 Flag-tagged BCL2L10. The cell lysates were prepared, 25 μg of total protein was loaded in each lane, and blots were made. Overexpressed BCL2L10 was visible by the antibody against Flag. Purified recombinant HIP1R protein (upper panel) or BSA were incubated with the renatured blot, and Western blotting was performed with the HA antibody. The membrane incubated with recombinant HIP1R protein was stripped and detected for BCL2L10 and GAPDH with antibodies against Flag or GAPDH, respectively. Exactly the same amount of mammalian total lysates and recombinant HIP1R produced at the same time were used in each lane.
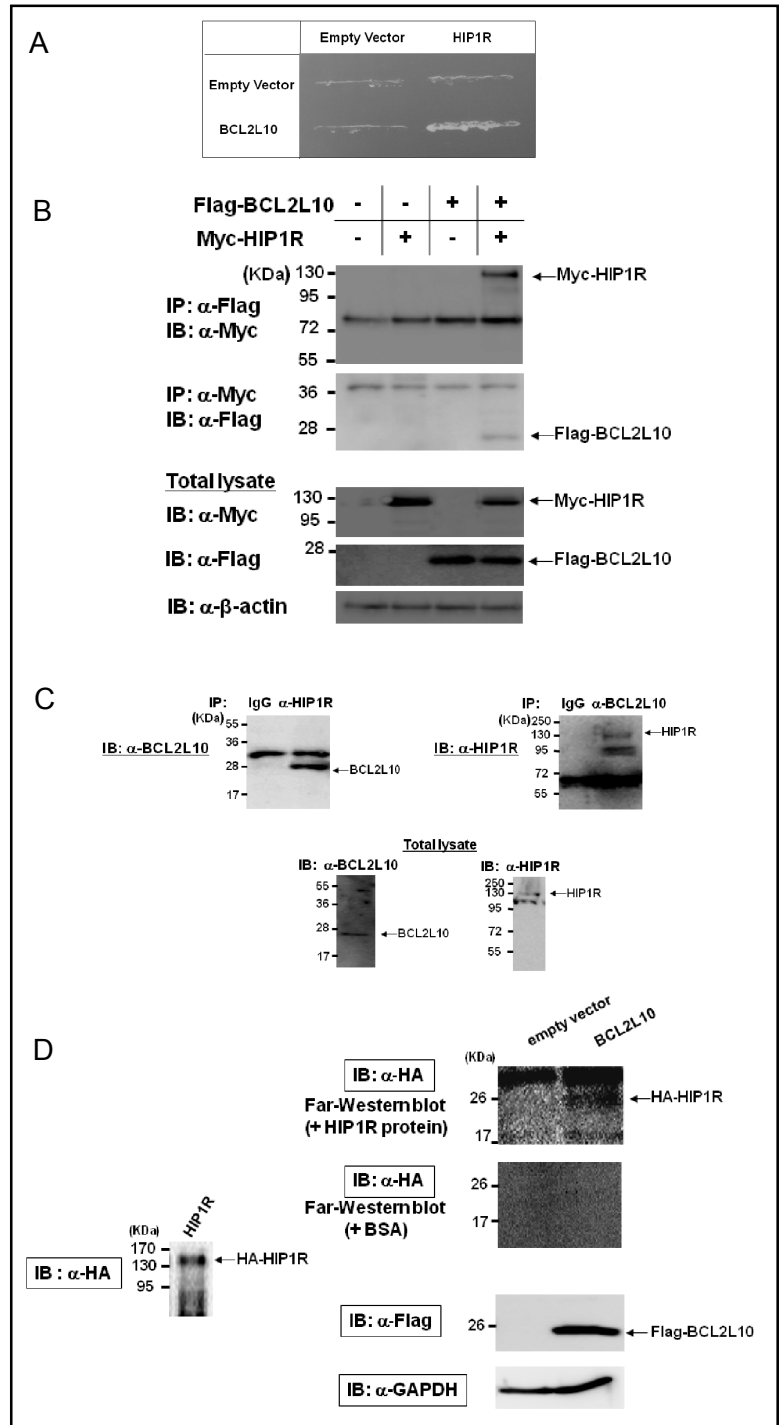
Results

BCL2L10 interacts with HIP1R

After identification of HIP1R as an interacting protein of mouse Diva from a rat ovarian cDNA library via yeast two-hybrid screening, the human homologues of the two proteins, BCL2L10 and HIP1R, were cloned and their specific interaction was further determined in yeast. As shown in Figure 1A, BCL2L10 fused with GAL4 DNA binding domain interacted with HIP1R containing the activating domain of GAL4. The *in vivo* interaction of BCL2L10 and HIP1R in mammalian cells was determined by immunoprecipitation. Overexpressed Flag-tagged BCL2L10 protein was co-immunoprecipitated with Myc-tagged HIP1R using an anti-HA monoclonal antibody (Fig. 1B). Conversely, HIP1R was also efficiently immunoprecipitated with ectopically expressed Flag-tagged BCL2L10 protein pulled down using the anti-

Fig. 2. Truncations of both the ANTH and THATCH domains of HIP1R greatly attenuated the interaction with BCL2L10. (A) Different HIP1R mutants (ΔANTH, ΔTHATCH, ΔANTHΔTHATCH and ΔCoiled-coil) lacking domains were generated to delineate the BCL2L10-binding region of HIP1R. (B) 293T cells (3.0 x 10⁶) were transfected with 3 μg of pcDNA3 Flag-tagged BCL2L10 along with Myc-tagged HIP1R, ΔANTH, ΔTHATCH, ΔANTHΔTHATCH or ΔCoiled-coil and incubated for 24 h. The cell lysates were immunoprecipitated with either anti-Flag or anti-Myc antibodies, and the immunoprecipitates were Western blotted using anti-Myc or anti-Flag antibodies, respectively. The expression of the mutants was confirmed by immunoblot using anti-Myc antibodies.
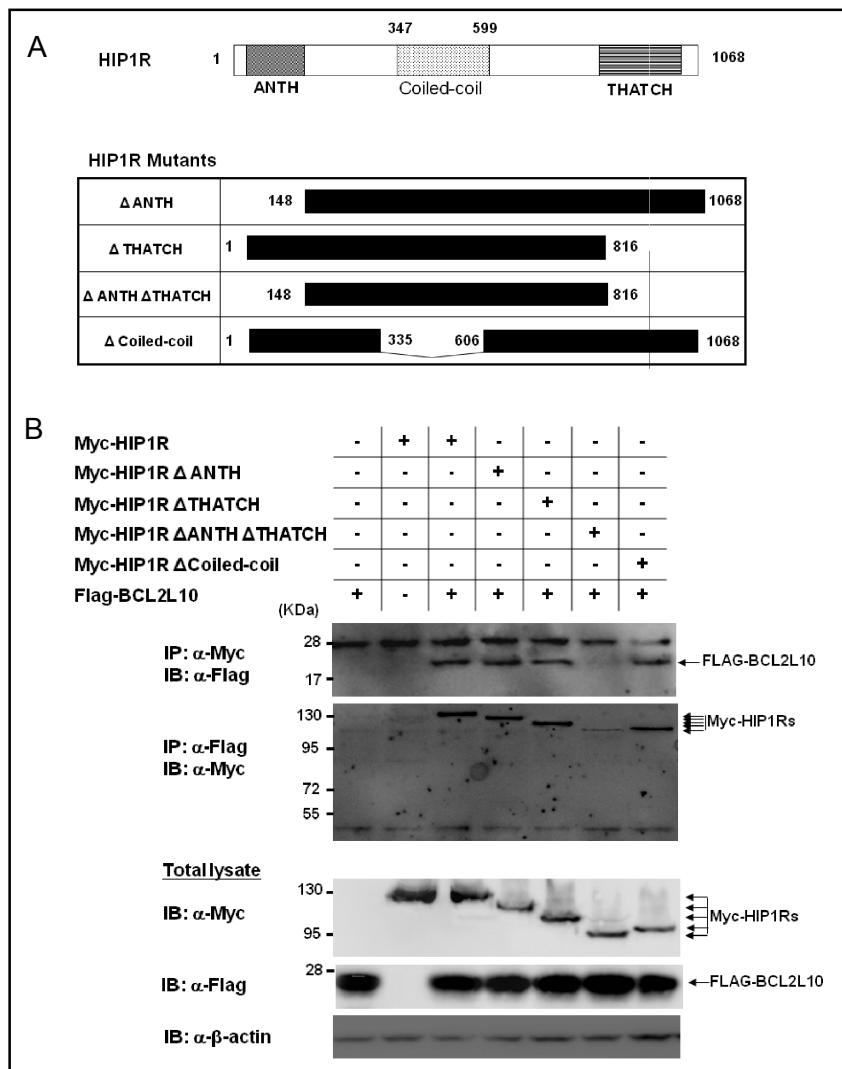

Flag polyclonal antibody (Fig. 1B). *In vivo* association of endogenous HIP1R and BCL2L10 proteins was confirmed by the co-immunoprecipitaion of HIP1R and BCL2L10 in 293T cells (Fig. 1C). To address whether the interaction between HIP1R and BCL2L10 is due to a direct association between two proteins, we attempted to produce their recombinant proteins. The recombinant protein of HIP1R was purified, while we were unsuccessful to purify BCL2L10 (data not shown). Alternatively, we performed the Far-Western blot analysis. Overexpressed Flag-BCL2L10 protein from 293T cells was separated by SDS-PAGE, transferred to a membrane. After renaturing proteins in the membrane which is subsequently incubated with recombinant HIP1R protein or BSA as a negative control. The BCL2L10-bound recombinant HIP1R protein was visible after a detection using the HA antibody (Fig. 1D; upper panel), pointing the direct interaction between BCL2L10 and HIP1R.

Deletion of both ANTH and THATCH domains of HIP1R greatly diminished the interaction with BCL2L10

Because HIP1R has distinct domains that direct its interactions with other proteins, we tried to determine the domain of HIP1R responsible for its interaction with BCL2L10. Truncated HIP1R mutants were generated, and their binding ability toward BCL2L10 was tested by immunoprecipitation. HIP1R is comprised of ANTH, a central coiled-coil domain containing leucin zipper, and THATCH domains as shown in Figure 2A. HIP1R mutant proteins that lack either the ANTH domain (ΔANTH), the THATCH domain (ΔTHATCH), or the coiled-coil region (Δcoiled-coil) were efficiently immunoprecipitated with BCL2L10 (Fig. 2B), suggesting that the truncations of one of ANTH, THATCH, or coiled-coil domains of HIP1R do not affect its association with the BCL2L10 protein, significantly. In contrast, The HIP1R mutant

Fig. 3. HIP1R activates caspase 9 and disrupts the mitochondrial membrane potential. (A) 293T cells (1.0 x10^6) were transfected with 3.0 μg of pcDNA3 empty vector (lane #1), Flag-BCL2L10 (1.5 and 3.0 μg) (lane #2-3), Myc-HIP1R (0.3, 0.9, 1.5 and 3.0 μg) (lane #4-7), and Myc-HIP1R (0.3, 0.9 and 1.5 μg) together with 1.5 μg of Flag-BCL2L10 (lane #8-10). The cell lysates were prepared after 24 hours of incubation. Activation of caspases was assessed by immunoblot analyses with anti-caspase 9, anti-caspase 3, and anti-caspase 8 antibodies. Expression of Flag- BCL2L10 and Myc-HIP1R proteins were determined by Western blots with anti-BCL2L10 or anti-HIP1R antibodies, respectively. Equal loading of the lysates was confirmed by Western blot analyses using the anti-β-actin antibody. (B) 293T cells (1.0 x10^6) were transfected with 3.0 μg of pcDNA3 empty vector, HA-HIP1R, Flag-BCL2L10, and BAK, or 1.5 μg each of pcDNA3 HA-HIP1R and Flag-BCL2L10. For the caspase inhibitor treatment, the cells were incubated with z-VAD-fmk (10 μM) at 6 h post-transfection. Twenty four hours after transfection, cell lysates were prepared, subjected to electophoresis, and immunoblotted with respective antibodies. (C) 293T cells (1.0 x10^6) were transfected with 3 μg of pcDNA3 empty vector, HA-HIP1R, Flag-BCL2L10, BAK, or 1.5 μg each of pcDNA3 HA-HIP1R and Flag-BCL2L10. Changes in mitochondrial membrane potential (MMP) were measured by FACS analysis after incubation of the cells with JC-1. Data are from three independent experiments (mean ± S.D.) and presented as percent of MMP relative to the empty vector control. Asterisks represent statistical significance relative to the control (p<0.05).
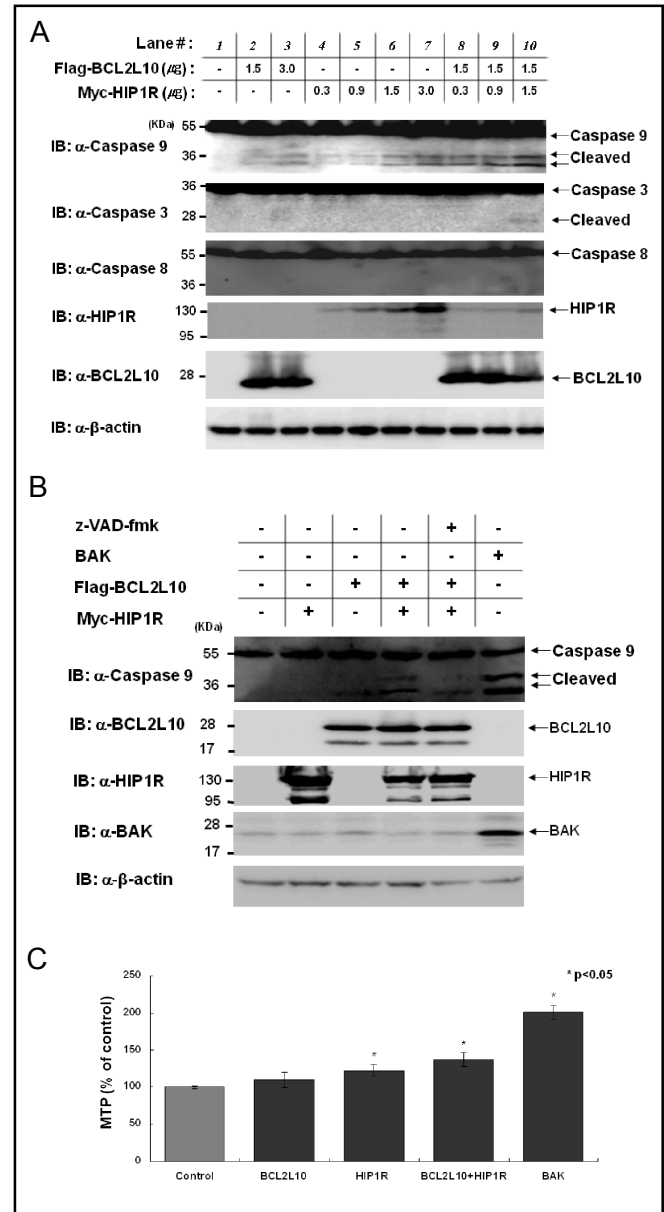
lacking both ANTH and THATCH domains (ΔANTHΔTHATCH) had a greatly compromised interaction with BCL2L10 as their interaction was barely detectable in the pull-down assay (Fig. 2B). The deletion of ANTH and THATCH domains could possibly lead to a conformational change, improper localization or other conditions, which eventually results in the compromised association with BCL2L10.

HIP1R induces caspase 9 activation

Since we observed the interaction of HIP1R with a member of the apoptosis-regulating family, we assessed whether HIP1R itself can activate caspases. Plasmids encoding HIP1R or BCL2L10 were overexpressed in 293T cells, and the lysates were produced after 24 h of incubation. As shown in Figure 3A, increasing expression of HIP1R protein led to the activation of caspase 9 in a dose-dependent manner, while no obvious active forms of caspase 9 was detected in the control cells transfected with an empty vector. The activation of caspase 9 was also observed with ectopic expression of BCL2L10 and the response was augmented after co-expression of HIP1R (Fig. 3A). In addition, cleaved caspase 3 was detectable after co-expression of HIP1R and BCL2L10, while no significant activation of caspase 8 was observed by Western blot analyses (Fig. 3A). The caspase 9 activation induced by co-expressions of BCL2L10 and HIP1R was less than the one elicited by BAK overexpression and was partially blocked by the presence of a pan-caspase inhibitor, z-VAD-fmk, (Fig. 3B).

HIP1R dissipates cellular mitochondrial membrane potential

The Bcl-2 family regulates apoptotic events that involve mitochondrial outer membrane permeabilization
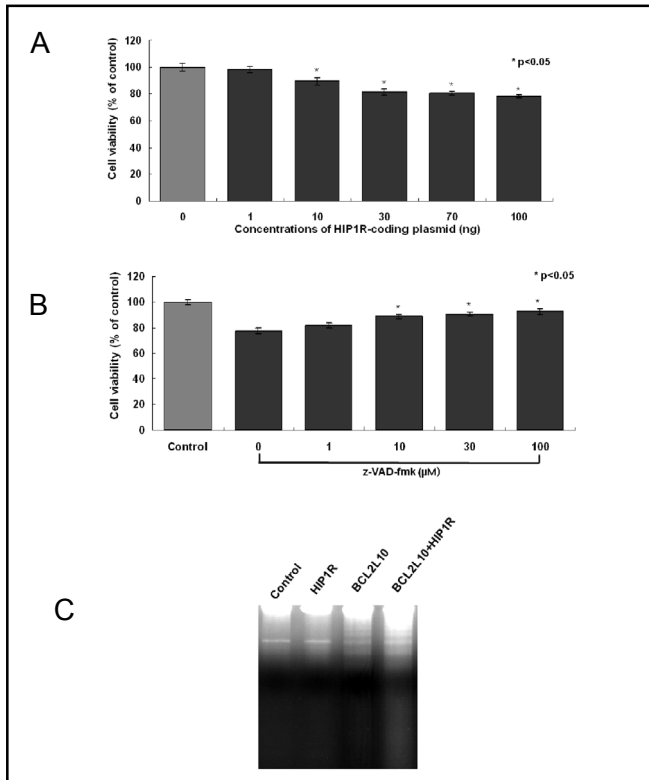
Fig. 4. HIP1R induces caspase-mediated cell death. (A) Cell viability assays were performed using 293T cells (3.5×10^4) after transfection with increasing amounts of pcDNA3 HA-HIP1R. At zero concentration, the empty vector (100 ng) was used. Three independent experiments, in duplicate, were performed. Data are expressed as the percentage (mean ± S.E.M) of viable cells relative to the control. Asterisks represent statistical significance relative to the control ($p<0.05$). (B) Cell viability was assessed as shown in (A) except that the cells were transfected with 100 ng of pcDNA3 HA-HIP1R and incubated with different concentrations of z-VAD-fmk. Asterisks indicate statistically significant values relative to the control ($p<0.05$). (C) 293T cells (1.0×10^6) were transfected with an empty vector (3.0 μg), Flag-BCL2L10 (3.0 μg), Myc-HIP1R (3.0 μg) or both (1.5 μg each). The DNA fragmentation was visualized by staining with ethidium bromide.

(MOMP) and thus the changes in MMP that occur during apoptosis [28]. Using the metachromatic fluorochrome JC-1 dye [29, 30], the dissipation of MMP was measured. Forced expression of HIP1R significantly disrupted MMP in 293T cells, but the response was moderate compared to the one elicited by Bak overexpression (Fig. 3C). Although no apparent change in MMP by ectopic expression of BCL2L10 was observed, the co-expression of BCL2L10 with HIP1R caused a distinct MMP dissipation (Fig. 3C).

HIP1R induces caspase-mediated cell death

Overexpression of HIP1R induced cell death in 293T cells in a dose-dependent manner, but this cell killing effect was not drastic (Fig. 4A). The incubation with increasing concentrations of z-VAD-fmk, a pan-caspase inhibitor, attenuated the cell death elicited by HIP1R (Fig. 4B) suggesting that HIP1R-induced cell death entails caspase(s) function, at least in part. Because Hoechst staining of the cells revealed that HIP1R expression resulted in discrete chromatin condensation and nuclear fragmentation from a small population of 293T cells (data not shown), DNA fragmentation assays were performed in cells after overexpression of HIP1R, BCL2L10 or both proteins. As shown in Figure 4C, a significant increase of fragmentation of DNA was not obvious after overexpression of HIP1R compared to the control although HIP1R clearly increased DNA fragmentation occurred by BCL2L10. Considering the abilities of HIP1R to induce MMP dissipation, caspase activation, and cell death although these effects were not extensive, the DNA fragmentation assay we performed seems to be less sensitive method to assess HIP1R response.

HIP1R increases the association of BCL2L10 with caspase 9

Since the mouse ortholog of BCL2L10, Diva, forms a ternary complex with overexpressed caspase 9 and Apaf-1 [5, 6], we assessed whether BCL2L10 is also able to interact with the apoptosome. As shown in Fig. 5, BCL2L10 was efficiently immunoprecipitated with endogenous caspase 9, whereas binding of HIP1R to caspase 9 was not detectable in this cellular context (Fig. 5). Next, we examined any influence of HIP1R on the complex formation of BCL2L10. Interestingly, in the presence of ectopically expressed HIP1R, the association of BCL2L10 and caspase 9 was significantly increased as determined by pull-down experiments with either anti-caspase 9 or anti-Flag antibodies (Fig. 5). We neither observed the association of endogenous Apaf-1 with HIP1R nor with BCL2L10 (data not shown), but we are not confident at this point whether it is due to the absence of their interaction or because of low level of endogenous Apaf-1 that hinders the pull-down assays.

BAK is required for HIP1R-induced cell death

To further delineate the mechanism by which HIP1R induces apoptosis, we assessed its cell death response in bax$^{-/-}$, bak$^{-/-}$, and bax$^{-/-}$bak$^{-/-}$ MEF cells. Overexpression of HIP1R in wild-type MEF cells exhibited more profound cell death compared to the one in 293T cells (Fig. 6),
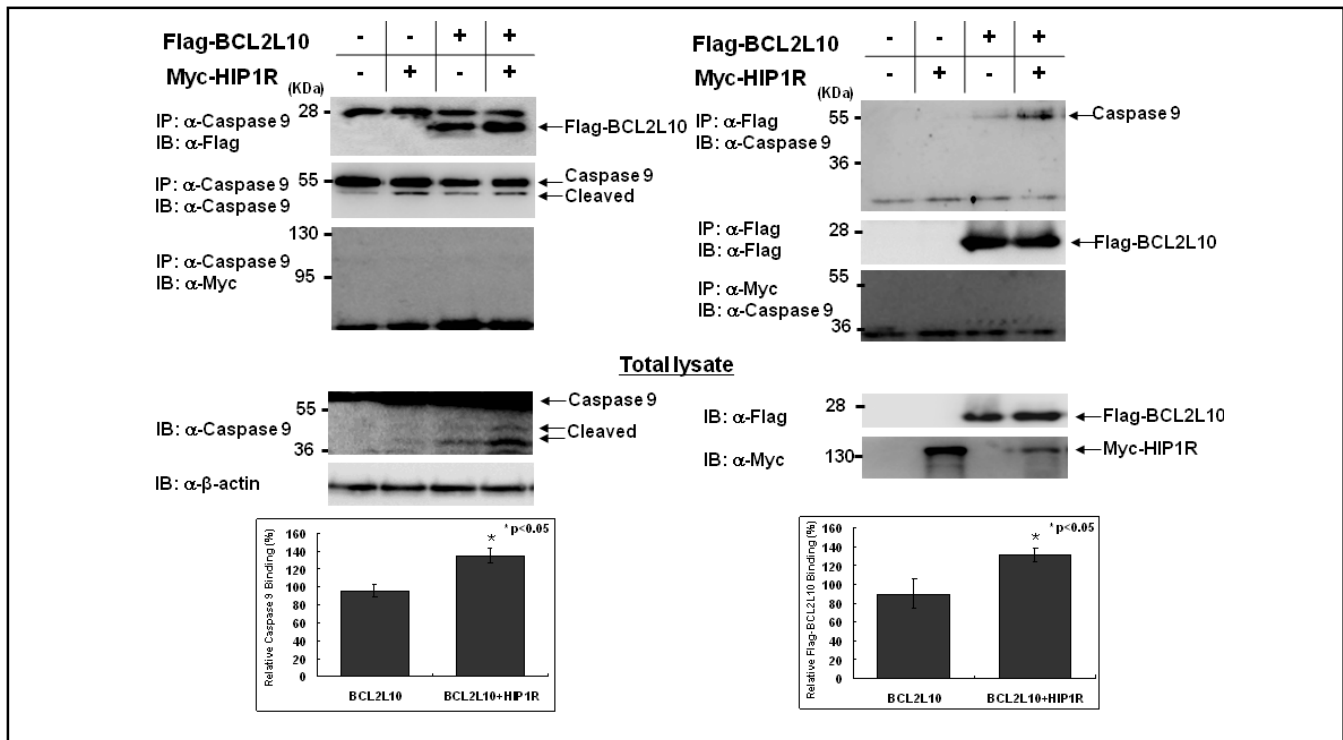
Fig. 5. BCL2L10 associates with endogenous caspase 9, which is augmented by HIP1R. 293T cells (3.0×10^6) were transfected with 3 μg of pcDNA3 Flag-BCL2L10, Myc-HIP1R, or both and incubated for 24 h. As a control, cells were transfected with the pcDNA3 empty vector (6 μg). Following IP, Western blots were performed and the immunoprecipitated proteins were detected with anti-Flag, anti-Myc, and anti-caspase 9 antibodies. Relative caspase 9 binding (%) in the left panel represents the portion of BCL2L10-bound caspase 9 immunoprecipitates (IP: anti-caspase 9, IB: anti-Flag) from total caspase 9 immunoprecipitates (IP: anti-caspase 9, IB: anti-caspase 9). Relative Flag-BCL2L10 binding (%) in the right panel represents the portion of caspase 9-bound BCL2L10 immunoprecipitates (IP: anti-Flag, IB: anti-caspase 9) from total Flag-BCL2L10 immunoprecipitates (IP: anti-Flag, IB: anti-Flag). The relative bindings in cells with Flag-BCL2L10 overexpression were considered 100%. Data are from three independent experiments and are expressed as mean ± S.D. Asterisks indicate a significant value compared to the results followed by the overexpression of BCL2L10 alone ($p < 0.05$).

suggesting MEF cells are more susceptible for HIP1R-induced apoptosis than 293T cells. However, strikingly, HIP1R completely failed to exhibit cell killing effect in the $bax^{-/-}bak^{-/-}$ double knockout cells. The forced expression of HIP1R completely failed to induce cell death in Bak-deficient cells (Fig. 6). In contrast, HIP1R-induced cell killing effect was not altered in Bax-depleted cells (Fig. 6). Collectively, these results clearly demonstrate that HIP1R-mediated cell death requires functional Bak protein, implying that Bak is an important mediator of HIP1R response in this circumstance.

Discussion

In the present study, we provide a new signaling network of HIP1R and BCL2L10 as HIP1R interacted with a member of Bcl-2 family, BCL2L10 under the endogenous condition. In addition, the ectopic expression of HIP1R resulted in moderate MMP dissipation, caspase 9 activation, and cell death. The co-expression of BCL2L10 did not seem to critically affect these responses, except for some additive effects (Fig. 3). Therefore, in this cellular context, we demonstrated that HIP1R induces modest apoptosis, but the significance of the interaction between HIP1R and BCL2L10 proteins under physiological conditions is unclear at this point and requires follow-up studies. Presence of other undiscovered functional networks between these two proteins could also be possible.

HIP1R and HIP1 are the only members of the HIP1 family found in mammals [19]. Both proteins share 49% identical sequences and exhibit both common and unique cellular properties. Although the influences of HIP1 on
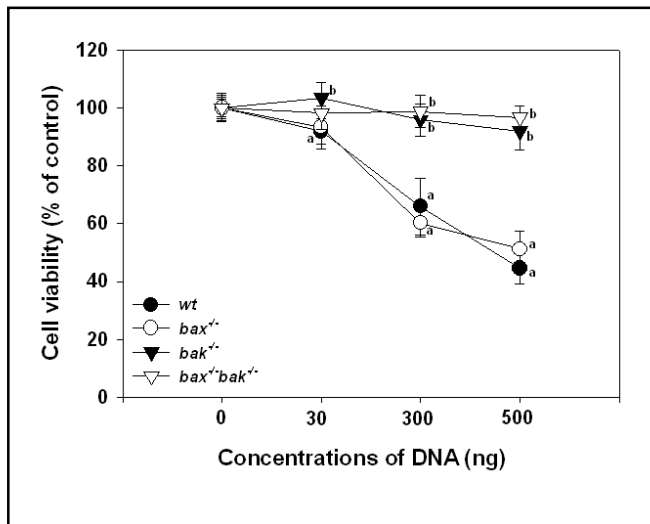
Fig. 6. The proapoptotic Bcl-2 protein Bak is pivotal in HIP1R-induced cell death. Wild-type and knockout MEF cells for bak$^{-/-}$, bax$^{-/-}$ and bax$^{-/-}$bak$^{-/-}$ were transfected with increasing concentrations of pcDNA3 HIP1R, and cell viability was measured after 24 h of incubation. At zero concentration of DNA, pcDNA3 empty vector (500 ng) was transfected to four different cell lines as controls. Data are expressed as the percentage (mean ± S.D.) of viable cells relative to the control group. Significant values relative to each control cells transfected with the empty vector or wild-type (wt) cells at each respective concentration were indicated with a or b respectively (p<0.01). All data are from three independent experiments performed in duplicate.

cell survival and death are not uniformly consistent, overexpression of HIP1 induces apoptosis in different cells [22-25]. In contrast, only few previous studies are available regarding the role of HIP1R on apoptosis. The induction of apoptosis by the epsin N-terminal homology (ENTH) domain-deleted HIP1R mutant, but not by the full length HIP1R, has been reported [19].

HIP1 has been known to directly bind to caspase 9 and Apaf-1 and activates caspase 9 in rat neuronal cells [25]. Diva or Boo, the mouse ortholog of BCL2L10, also associates with overexpressed caspase 9 and Apaf-1, the components of apoptosome [4, 5]. Thus, we assessed the role of HIP1R regarding the complex formation with apoptosome constituents. We for the first time found the association of BCL2L10 with caspase 9 in 293T cells even without its overexpression, and, strikingly, the association was significantly elevated by increasing expression of HIP1R (Fig. 5). Even though the underlying mechanism is uncertain at this point, the augmented

BCL2L10-mediated activation of caspase 9 after HIP1R overexpression (Fig. 3A and B) maybe partly related to the increased binding between BCL2L10 and caspase 9 upon the forced expression of HIP1R. Unveiling the detailed roles of HIP1R and BCL2L10 in the regulation of apoptosome would be of interest.

The members of Bcl-2 family are central regulators of apoptosis in cells, and proapoptotic Bax and Bak are executors of the intrinsic cell death pathway as they act on the mitochondrial outer membrane by forming a pore followed by MOMP and cytochrome c release. Thus, we determined the dependency of HIP1R on Bax and/or Bak for its cell death function using the knockdown cells of bax$^{-/-}$, bak$^{-/-}$, and bax$^{-/-}$bak$^{-/}$ and found that Bak is indispensable for HIP1R-induced cell killing effect while Bax is dispensable (Fig. 6). Therefore, these data imply that HIP1R-induced cell death is largely, if it is not all, mediated by the intrinsic cell death pathway involving cellular mitochondria.

Nakajima et al [31] unveiled a striking function of BNIP1, a member of proapoptotic BH3-only subfamily. They demonstrated that BNIP1 is in a complex with syntaxin 18 and necessary for the maintenance of the endoplasmic reticulum network structure, demonstrating that a Bcl-2 family member has an additional function beyond its role in mitochondrial apoptotic signaling. Accordingly, the physical interaction observed between BCL2L10 and HIP1R from the present study also provides a previously undiscovered role for a member of Bcl-2 family, as the major known functions of HIP1R are in the regulation of endocytosis and actin assembly [14-16]. Therefore, it is possible that BCL2L10 may play a role in HIP1R-mediated endocytic and actin machinery, and future studies to uncover the nature of this signaling would be valuable.

In summary, this study provides a novel and direct connection between HIP1R and BCL2L10 in apoptosis signaling. Furthermore, we demonstrated that HIP1R is a modest apoptotic molecule that requires BAK to exert its cell death function and enhances the association of BCL2L10 and caspase 9.

Acknowledgements

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