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RESEARCH ARTICLE

Human Bop is a novel BH3-only member of the Bcl-2 protein family

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ABSTRACT

One group of Bcl-2 protein family, which shares only the BH3 domain (BH3-only), is critically involved in the regulation of programmed cell death. Herein we demonstrated a novel human BH3-only protein (designated as Bop) which could induce apoptosis in a BH3 domain-dependent manner. Further analysis indicated that Bop mainly localized to mitochondria and used its BH3 domain to contact the loop regions of voltage dependent anion channel 1 (VDAC1) in the outer mitochondrial membrane. In addition, purified Bop protein induced the loss of mitochondrial transmembrane potential (ΔΨm) and the release of cytochrome c. Furthermore, Bop used its BH3 domain to contact pro-survival Bcl-2 family members (Bcl-2, Bcl-X_L, Mcl-1, A1 and Bcl-w), which could inhibit Bop-induced apoptosis. Bop would be constrained by pro-survival Bcl-2 proteins in resting cells, because Bop became released from phosphorylated Bcl-2 induced by microtubule-interfering agent like vincristine (VCR). Indeed, knockdown experiments indicated that Bop was partially required for VCR induced cell death. Finally, Bop might need to function through Bak and Bax, likely by releasing Bak from Bcl-X_L sequestration. In conclusion, Bop may be a novel BH3-only factor that can engage with the regulatory network of Bcl-2 family members to process intrinsic apoptotic signaling.

KEYWORDS apoptosis, BH3 domain, Bop

INTRODUCTION

The Bcl-2 family consists of divergent and interactive members that regulate programmed cell death by mechanisms evolutionarily conserved from nematodes to humans (Cory and Adams, 2002; Danial and Korsmeyer, 2004; Wong and Puthalakath, 2008). In mammalian cells, the pro-survival members of the Bcl-2 family (Bcl-2, Bcl-X_L, Mcl-1, A1, Bcl-w) share four Bcl-2 homology (BH) domains, namely BH1-4 (Kelekar and Thompson, 1998; Giam et al., 2008). They are often counteracted by two groups of pro-apoptotic Bcl-2 family members, i.e., the Bax and the BH3-only sub-groups. The former sub-group members (Bak, Bax, Bok/Mtd), whose structures are related to their pro-survival counterparts, possess three out of four BH regions (BH1-3) (Suzuki et al., 2000; Moldoveanu et al., 2006). The BH3-only members share a minimal sequence homology in the amphipathic α-helical BH3 domain. The best characterized BH3-only proteins include Bim, Bad, Bid, Bik/Nbk, Bmf, Noxa, Puma, and Hrk/DP5 (Happo et al., 2012), whereas more BH3-only like proteins have thus far reported, including Beclin1, Bnip3, Bnip3L/Nix, Rad9, MAP-1, SphK2, BRCC2, TG2, Mule, HER4, HER2, ApoL6 and ApoL1 (Lomonosova and Chinnadurai, 2008).

Biochemical and genetic experiments indicate that BH3-only proteins play a pivotal function in response to a variety of cytotoxic stimuli (Willis and Adams, 2005; Vo and Letai, 2010). The conventional model suggests that Bid, Bim and Puma may function as “activators” to directly trigger oligomerization and activation of Bak or Bax to induce

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cytochrome c release (Letai et al., 2002; Kuwana et al., 2005), whereas other BH3-only proteins, like Bad or Bik, may function as "sensitizers" to promote apoptosis by antagonizing pro-survival Bcl-2 proteins to release "activators" BH3-only proteins (Letai et al., 2002; Chen et al., 2005; Kim et al., 2006). In another model, Bak is kept inactive by either Mcl-1 or Bcl-X_L confinement (Willis et al., 2005) or voltage dependent anion channel 2 (VDAC2) restriction (Cheng et al., 2003) in resting cells. Therefore, activation of Bak or Bax usually requires BH3 peptides of the BH3-only proteins to directly contact pro-survival Bcl-2 proteins or VDACs to free up Bak or Bax . Once activated, Bak or Bax form oligomers in mitochondrial outer membrane to facilitate membrane permeabilization and release of pro-apoptotic factors, such as cytochrome c, for downstream caspase activation and initiation of cell death (Giam et al., 2008; Leber et al., 2010; Shamas-Din et al., 2011).

Herein we report another putative human BH3-only protein (designated as Bop) that can induce apoptosis in a BH3-domain dependent manner. Further analyses show that Bop may localize to outer mitochondrial membrane via VDAC1 and serves as a functional component of Bcl-2 regulatory network in the process of intrinsic apoptotic signaling.

RESULTS

Bop is a putative BH3-only protein

To search for novel Bcl-2 family members, we used the BH3 sequence (LAQIGDEMD) of mouse Bid as a query to blast GenBank databases. Besides Bid and Bcl-w, we also scored a hypothetical human protein C22orf29 (*aka* FLJ21125, GenBank accession: NP_078903.3) with an identical BH3 sequence (Fig. 1A). Further BLAST search revealed the presence of Bop orthologs in chimpanzee (similarity 98%), Sumatran orangutan (94%), white-tufted-ear marmoset (87%), rabbit (71%) and horse (67%), all of which contain the highly conserved BH3 domain (Fig. S1). Surprisingly, no Bop homolog can be retrieved from mouse genome. We thereafter designated C22orf29 as a putative BH3-only protein (Bop). Bop was 364 amino acids in length without trans-membrane domain. The secondary structure as predicted by PSIPRED (Jones, 1999) showed that Bop was a very flexible protein containing only 23% α-helix and 2% β-strands (Fig. S1), and the BH3 region exhibited an intact α-helical folding conserved in entire Bcl-2 family members (Petros et al., 2004; Hinds and Day, 2005). Circular dichroism analysis confirmed that Bop contained about 25% helix and 5% β-strands (data not shown). In good agreement with the previous results (Strausberg et al., 2002), Northern blotting showed that Bop mRNA, which was about 6.5 kb in length, was universally expressed in all tissues tested (Fig. 1B), indicative of its fundamental cellular function. Western blotting using mouse antisera could detect Bop in A549, HeLa and 293T cells, with apparent relative molecular mass around 40 kDa (Fig. 1C). As expected, murine embryonic fibroblast cells did not show any expression of Bop.

Bop induces cell death in both time- and dose-dependent manners

To test whether Bop was indeed pro-apoptotic, we first overexpressed the GFP-tagged Bop (GFP-Bop) in HeLa cells to measure the cell survival rates. GFP positive cells gated by flow cytometry were assessed for apoptosis (annexin V-Cy5 positivity) (Fig. S2). The results showed that transiently expressed Bop was able to accelerate cell death in time- (Fig. 1D) and dose-dependent (Fig. 1E) manners. To determine whether the putative BH3 domain was required for Bop function, we deleted the entire BH3 domain (BopΔBH3) or mutagenized the conserved amino acid (L118 or D123) to alanine (Bop-L118A or Bop-D123A) within the putative BH3 domain of Bop (Fig. 2A). Transient transfection of these mutant derivatives in HeLa cells showed that truncation of BH3 domain or point mutation of L118 and D123 significantly reduced the pro-apoptotic activity of Bop (Fig. 2B). Therefore, these results suggested that Bop could induce apoptosis depending on its conserved BH3 domain.

Bop localizes to mitochondria and induces mitochondrial dysfunction depending on its BH3 domain

Because Bcl-2 family members modulate apoptosis primarily through action on mitochondrial components, we first set out to determine whether Bop possibly functioned through mitochondria. Confocal microscopic analysis showed that the endogenous Bop was expressed in cytoplasm showing a network-like distribution (Fig. S3). Similarly, GFP-Bop over-expressed in the cytoplasm of A431 cells displayed a punctate distribution that overlapped with mitochondria, while GFP-BopΔBH3 apparently lost the speckle distribution and became rather smear in cytosol (Fig. 3A). More careful quantitation by ImageStream analysis indicated that 37.6% of GFP-Bop precisely co-localized with the mitochondria (Fig. S4). These results illumined that Bop was able to reside on mitochondria dependent of the BH3 domain.

We then tried to determine whether Bop could affect mitochondrial membrane permeability. Because the full-length recombinant Bop was poorly expressed in *E.coli*, we deleted 61 amino acids of the N-terminus (BopΔN) which improved the expression efficiency and solubility (data not shown). BopΔN had the similar BH3-dependent pro-apoptotic activity to the full length version of Bop in cells (Fig. S5A and S5B). Incubation of GST-BopΔN caused abrupt dissipation of mitochondria transmembrane potential (ΔΨm), which was effectively inhibited by addition of recombinant GST-Bcl-X_L protein (Fig. 3B, top panel). GST-BopΔN was also able to

induce mild swelling of mitochondria (Fig. 3B, bottom panel). Perturbation of mitochondrial ΔΨm would induce release of apoptogenic factors. Indeed, cytochrome c release experiments showed that incubation of GST-BopΔN with isolated mitochondria readily induced cytochrome c release in a dose-dependent manner (Fig. 3C). Moreover, the BH3 domain was required for Bop to induce mitochondrial dysfunction since deletion of BH3 domain (GST-BopΔNΔBH3)

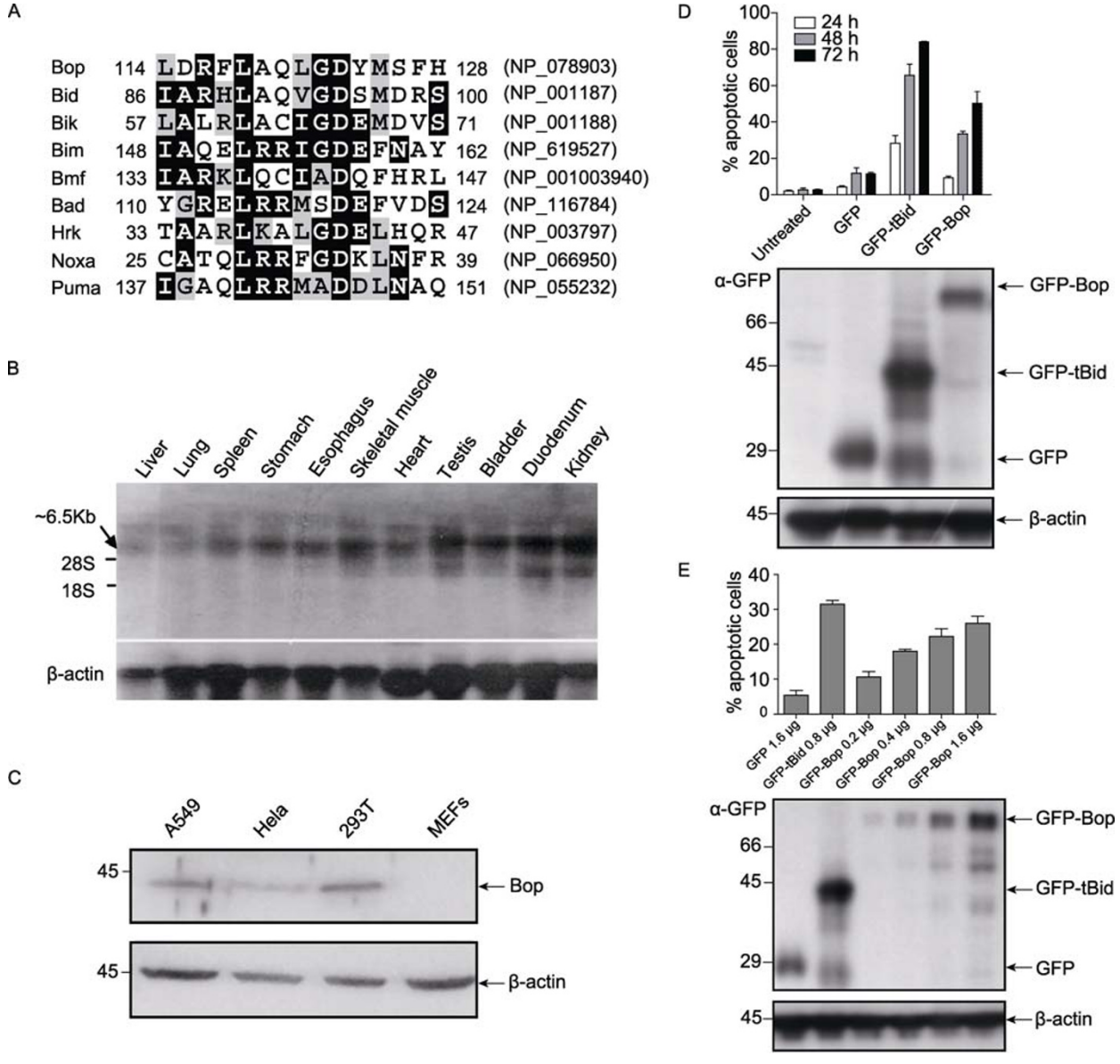

Figure 1. Bop contains a putative BH3 domain and induces apoptosis in time- and dose-dependent manners. (A) ClustalX alignment of the BH3 domains of Bop and other human BH3-only proteins sequences. Their accession numbers and amino acid coordinates are indicated. The amino acids in black and gray shades indicate identical and similar residues, respectively. (B) Ubiquitous expression of human Bop. Expression of Bop transcript among various human tissues is indicated at 6.5 kb, with 18S and 28S rRNA also indicated. The β-actin RNA was probed as the loading control. (C) The expression of Bop in whole cell lysates (100 μg proteins) from A549, HeLa, 293T and MEF cells was immunoblotted. Human Bop (~ 40 kDa) and the loading control of β-actin are indicated by arrows. (D) Bop induces cell death in a time-dependent manner. HeLa cells ($1x10^6$) transiently transfected with 1.6 μg pEGFP-N1-Bop, pEGFP-N1-tBid plasmid or parental plasmid pEGFP-N1 were gated for GFP-positive cells ($1x10^4$) by flow cytometry analysis. Annexin V-Cy5 and PI staining at indicated time points was analyzed. (E) Bop induces cell death in a dose-dependent manner. Apoptotic rates of HeLa cells transiently transfected with indicated amount of expression plasmids for GFP, GFP-Bop or GFP-tBid for 48 h were analyzed as in (D). In (D and E), expression of each ectopic protein was confirmed by immunoblotting with anti-GFP antibody. β-actin was immunoblotted as the loading control. The data represent the average of three independent experiments (mean ± s.e.m.).

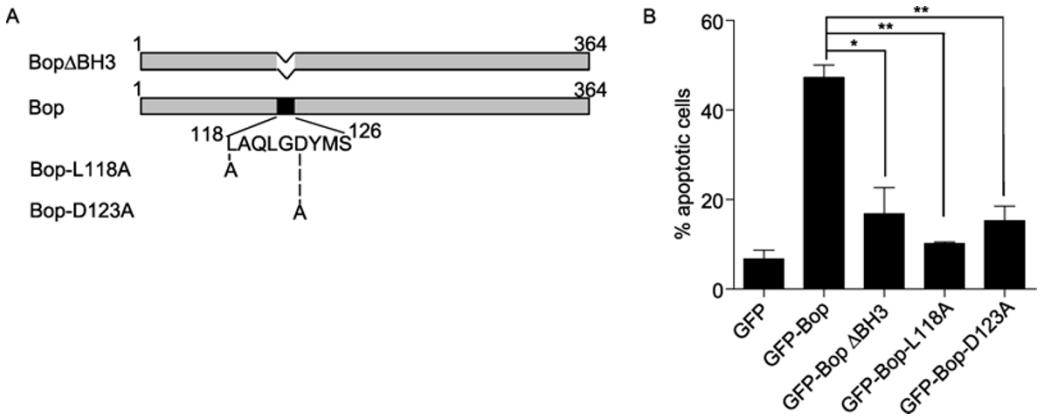

Figure 2. The BH3 domain is required for pro-apoptotic activity of Bop. (A) Schematic diagram of Bop in full length and BH3 domain deletion (BopΔBH3) as well as different mutant derivatives of Bop. (B) BH3 domain is required for Bop to induce cell death. Apoptosis of HeLa cells transiently transfected with 1.6 μg expression plasmids for GFP, GFP-Bop, GFP-BopΔBH3 or different mutant derivatives of Bop for 72 h was analyzed as in Fig. 1D. **P* < 0.05, ***P* < 0.01, *t* test.

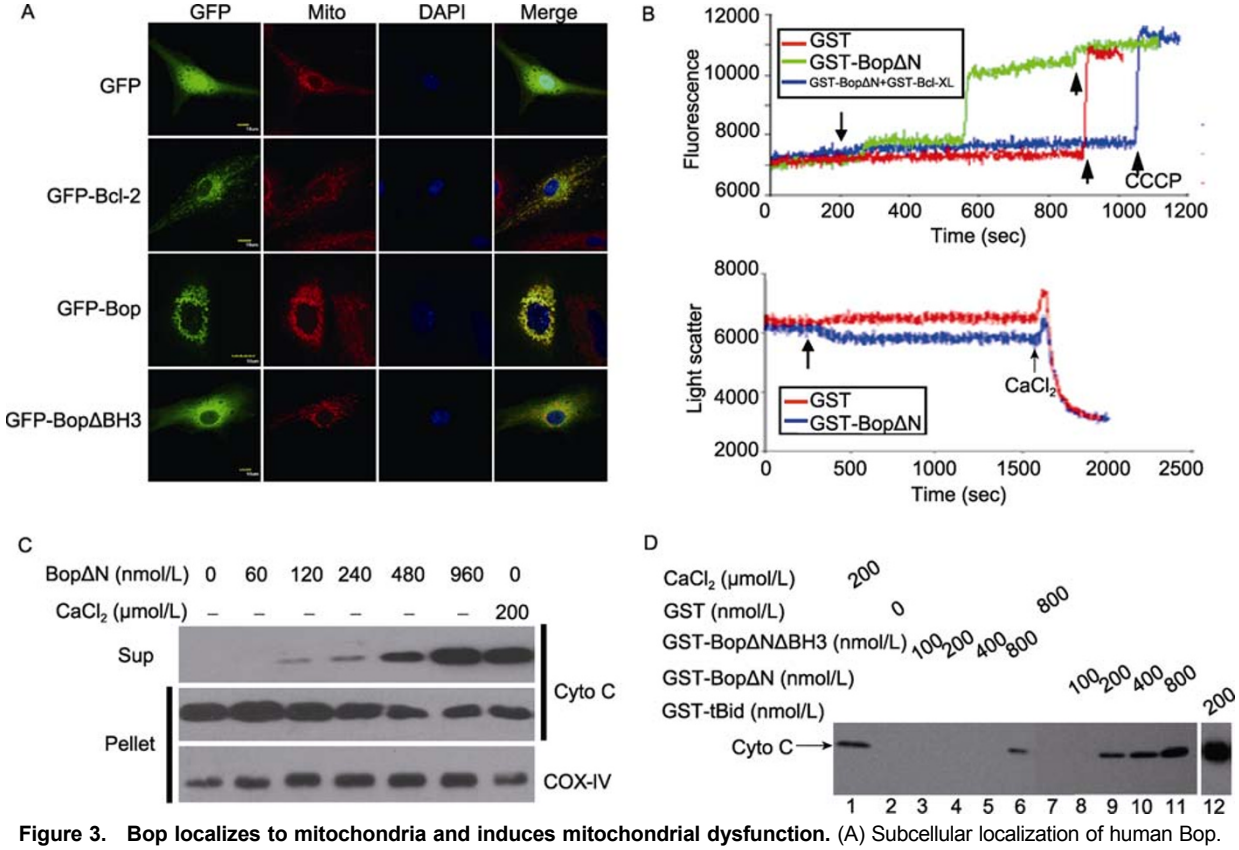

Figure 3. Bop localizes to mitochondria and induces mitochondrial dysfunction. (A) Subcellular localization of human Bop. GFP-tagged proteins as indicated were transiently transfected into A431 cells. 48h after transfection, cells were visualized by confocal laser scanning microscopy. Mitochondira were labeled with MitoTracker (Red) and nuclei were stained with DAPI (blue). GFP and GFP-Bcl-2 served as negative and positive controls, respectively. (B) Bop induces mitochondria dysfunction *in vitro*. *Top panel*: Loss of ΔΨm of mice liver mitochondria were analyzed after addition of GST-BopΔN (green), GST-BopΔN plus GST-Bcl-X_L (blue) and GST (red) at 200 s (downward arrow). Complete loss of ΔΨm was indicated by addition of CCCP (upward arrowheads). *Bottom panel*: Mitochondrial swell by the decrease of 90° light scattering was analyzed by adding GST-BopΔN (blue) and GST (red) to mitochondria at 200s (upward arrow). $CaCl_2$ was added at the end of experiments as a positive control for permeability transition pore opening (upward arrowhead). (C) Supernatants of mitochondria after incubation with indicated concentrations of recombinant BopΔN were analyzed by immunoblotting with antibody to cytochrome c (Cyto C). The mitochondrial pellets were immunoblotted with antibody to cytochrome c or cytochrome c oxidase subunit IV (COX-IV), respectively, for loading control. $CaCl_2$ was used as a positive control of cytochrome c release from mitochondria. (D) Dependence of BH3 domain for Bop to induce cytochrome c release was analyzed by incubating mitochondria with increasing concentrations of GST-BopΔN or GST-BopΔNΔBH3 protein. Cytochrome c release was measured as in (C), except that GST-tBid was used as a positive control.

almost abolished its ability to induce cytochrome c release (Fig. 3D, lanes 3–6). Therefore, Bop was able to localize to mitochondria and induce mitochondrial dysfunction. It is of note that Bop exerted a relatively weaker induction of cytochrome c release than tBid under this particular setting (Fig. 3D).

Bop interacts with VDAC1 using its BH3 domain

Previous studies by us and others (Shimizu et al., 1999; Shimizu et al., 2000; Shimizu et al., 2001; Shi et al., 2003; Zheng et al., 2004) indicate that VDACs regulate the release of apoptogenic factors from mitochondria to cytosol to activate the apoptotic cascade. To test whether VDACs could be involved in mitochondrial permeability change induced by Bop, we co-expressed HA-VDAC1 and myc-Bop or myc-BopΔBH3 in 293T cells. Co-immunoprecipitation experiments indicated that Bop but not BopΔBH3 associated with VDAC1 (Fig. 4A). GST pulldown assay using recombinant His-VDAC1and GST-BopΔN further showed that Bop was able to make direct protein-protein contact with VDAC1 (Fig. 4B). Furthermore, recombinant GST-Bop bearing point mutation at L118, but not Q120, G122 or D123, of the BH3 domain effectively interrupted the intra-molecular interaction between Bop and VDAC1 (Fig. 4B). As a control, GST-tBid or GST-Mcl-1 showed no interaction with VDAC1. Therefore, these results strongly indicated that Bop used its BH3 domain to specifically contact VDAC1. Furthermore, in such GST pull-down assays, pre-incubation of VDAC1 with antibody that targeting the cytosolic loop regions (mAb #20 and #25), but not the N- (mAb 31HL) or C-terminus (mAb #30) of VDAC1, could prevent Bop from binding to VDAC1 (Fig. 4C). This was reminiscent of previous findings that VDAC1 uses the loop regions to contact Bax (Shimizu et al., 2001; Shi et al., 2003) and Bim (Sugiyama et al., 2002) but not Bid (Shimizu et al., 2001; Weng et al., 2005) for the coordinated control of outer mitochondrial membrane permeability. Therefore, Bop may induce apoptosis by contacting VDAC1 and that leads to change of mitochondrial outer membrane permeability.

Bop interacts with pro-survival Bcl-2 family proteins using its BH3 domain

It has become clear that BH3-only proteins can, in most cases, promote cell death by antagonizing pro-survival Bcl-2 family members (Huang and Strasser, 2000). The aforementioned result that Bcl-X_L attenuated Bop-induced ΔΨm dissipation suggested a direct interaction between Bop and Bcl-X_L. To test whether pro-survival Bcl-2 proteins can inhibit Bop- induced apoptosis, we transiently co-expressed Bop with each pro-survival Bcl-2 protein in HeLa cells. Measurement of apoptosis rates by flow cytometry showed that Bop-driven apoptosis was partially alleviated by the presence of Bcl-w, Bcl-X_L, A1, Mcl-1, or Bcl-2 (Fig. 5A). Such a functional antagonism would suggest a physical interaction. We then over-expressed myc-Bop in 293T cells and co-immunoprecipitation showed that exogenous Bop could associate with endogenous Bcl-2, Bcl-X_L or Mcl-1 (Fig. 5B). Such a physical contact was further confirmed by

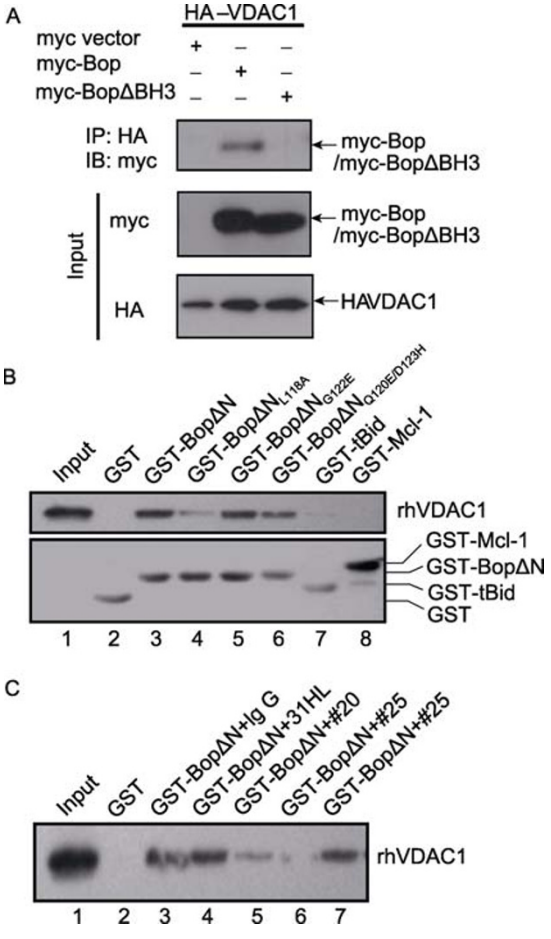

Figure 4. Bop interact with the loop regions of VDAC1. (A) Bop contacts VDAC1. Interaction between Bop and VDAC1 was analyzed by co-immunoprecipitation of 293T cell lysates transfected with pCMV-myc-Bop and pCMV-HA-VDAC1. (B) Recombinant VDAC1 physically contacts Bop. An equal amount (5 μg) of recombinant His-VDAC1 and GST-tagged proteins were mixed for GST pulldown and absorbed VDAC1 was immunoblotted with anti-His antibody (top). The requirement of BH3 domain for Bop to contact VDAC1 was also analyzed with different BopΔN mutant proteins. GST-tBid and GST-Mcl-1 were included in the assay as negative controls. The input proteins were assessed by immunoblotting using GST antibody (bottom) (C) Regions of VDAC1 to contact Bop were analyzed by the similar GST pulldown as in (B) except that various antibodies against N-terminus (31HL), loops (#20, #25) or C-terminus (#30) of VDAC1 were pre-incubated with the recombinant His-VDAC1. Non-specific mouse IgG was used as a sham.

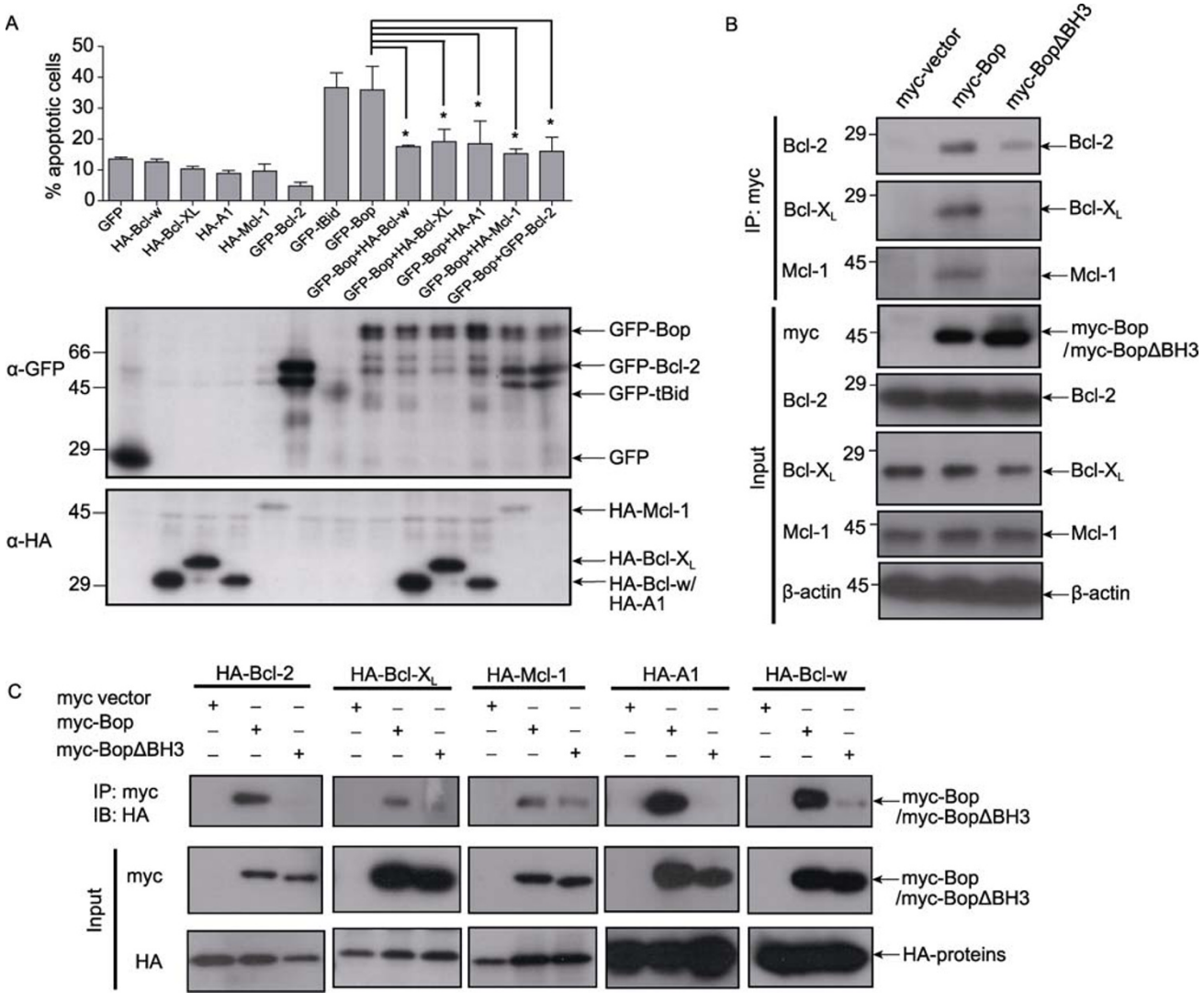

Figure 5. Pro-survival Bcl-2 proteins antagonize Bop by direct contact. (A) Apoptotic rates of HeLa cells overexpressing GFP-Bop in the presence of different pro-survival Bcl-2 proteins were analyzed by FACS as in Fig. 1D. The data represent the average of three independent experiments (mean ± s.e.m.). The expression levels of indicated proteins were measured by immunoblotting with antibody to GFP or HA tag (bottom). Co-immunoprecipitation assays of whole cell lysates of 293T cells transiently expressing indicated proteins show that Bop interacts with endogenous Bcl-2, Bcl-X_L or Mcl-1 (B) or each of the five pro-survival Bcl-2 members when co-expressed in 293T cells (C). The reliance on BH3 domain for inter-molecular interactions between Bop and each protein is also shown. The inputs of various proteins were analyzed by immunoblotting of cell lysates with antibody to myc or HA.

co-immunoprecipitation of each of pro-survival Bcl-2 members (HA-tagged) with co-expressed myc-Bop in 293T cells (Fig. 5C). Deletion of BH3 domain of Bop (myc-BopΔBH3), in contrast, destructed its interaction with these proteins (Fig. 5B and 5C), suggesting that the BH3 domain was required for Bop to contact these pro-survival Bcl-2 members. Therefore, these results suggested that pro-survival Bcl-2 family members may constrain and prevent Bop from inducing intrinsic apoptosis.

Overexpressed Bop and pro-survival Bcl-2 proteins antagonized each other in modulating apoptosis, suggesting that the molecular stoichiometry of Bop/Bcl-2 complex may play a role in determining cell fate. To test this hypothesis, we down-regulated endogenous Bop expression by siRNA. Bop deficiency selectively resisted to etoposide or vincristine (VCR) induced cell death (Fig. S6). Time course analysis also showed that VCR induced apoptosis in scrambled RNAi treated cells was significantly reduced in Bop RNAi cells (Fig. S6A), if ~50% of knockdown efficiency were normalized (Fig. S6A). VCR is a microtubule-interfering agent that induces

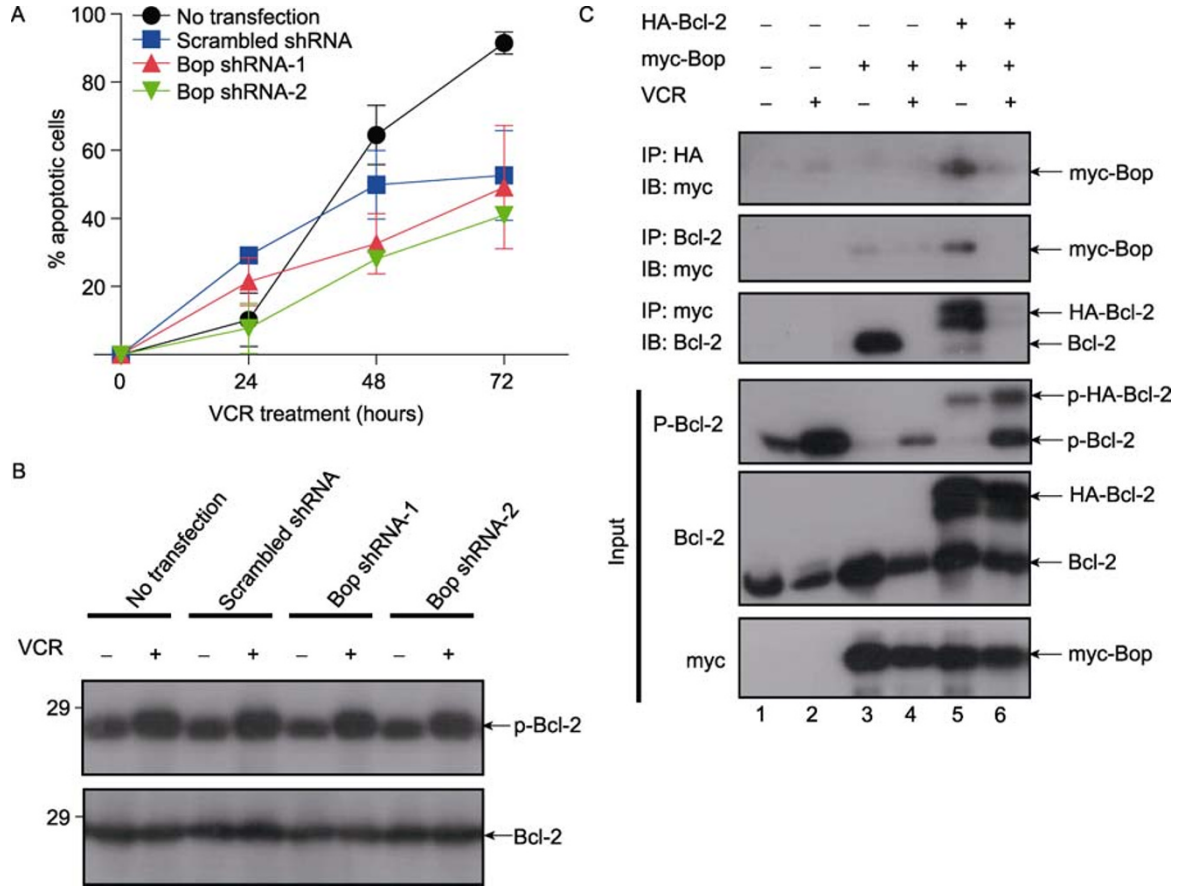

Figure 6. Bop is released from phosphorylated Bcl-2 in response to VCR induced cell death. (A) HeLa cells were transfected with pGPH1-Bop-shRNAs (#1 and #2) or scrambled shRNA for 72 h. Transfectant cells were then treated with VCR (400 ng/mL) for indicated time and cell death was determined by flow cytometry as in Fig. 1D. Percentage of apoptosis were means of three independent experiments (mean ± s.e.m.). (B) Bop knockdown does not affect Bcl-2 phosphorylation induced by VCR. HeLa cells with Bop knockdown were treated with VCR for 24 h as in (A), and levels of Bcl-2 phosphorylation (p-Bcl-2) and total Bcl-2 (Bcl-2) were determined by immunoblotting. (C) Inactivated Bcl-2 releases Bop. HeLa cells transiently expressing myc-Bop (lanes 3,4) or co-expressing myc-Bop and HA-Bcl-2 (lanes 5,6) were co-immunoprecipitated with antibodies to HA, Bcl-2 or myc, respectively after cells were treated with or without VCR (400 ng/mL) for 24 h. The input of Bcl-2, p-Bcl-2 and myc-Bop levels were shown by immunoblotting with equal loading of total proteins (~ 20 μg).

phosphorylation thus inactivation of Bcl-2 (Haldar et al., 1995; Yamamoto et al., 1999). Down-regulation of Bop did not interfere with either Bcl-2 expression or phosphorylation (Fig. 6B). Surprisingly, co-immunoprecipitaiton results showed that increased phosphorylation of endogenous (Fig. 6C, lane 4) or ectopically overexpressed (Fig. 6C, lane 6) Bcl-2 by VCR associated with less inter-molecular interaction with Bop. Therefore, these results suggested that, an appropriate apoptotic stimuli such as VCR, might be required to first disrupt Bcl-2 confinement on Bop and then release pro-apoptotic Bop for mitochondrial dysfunction and cell death.

Bop mediates apoptosis through Bak and Bax

Bak and Bax are essential to mediate apoptosis induced by BH3-only proteins (Zong et al., 2001; Jiang and Wang, 2004; van Delft and Huang, 2006). To test lastly whether Bop required Bak/Bax for function, we detected the interaction between Bop and Bak or Bax by co-immunoprecipitation experiments. The results showed that transiently co-expressed myc-Bop could associate with both Bak and Bax, albeit the requirement of BH3 was more obvious for Bop to contact Bak than Bax (Fig. 7A). We then asked whether Bax/Bak play any role in Bop-induced apoptosis. To do so, we transiently expressed myc-Bop in murine embryonic fibroblast (MEF) cells with both Bak and Bax genes knocked out (Bak/Bax dko). Flow cytometric analysis showed that, unlike wild type (wt) cells, Bak/Bax deficiency (dko) caused cells resistant to myc-Bop induced apoptosis (Fig. 7B). Likewise, mitochondria isolated from Bak/Bax dko cells could not be permeated by GST-BopΔN

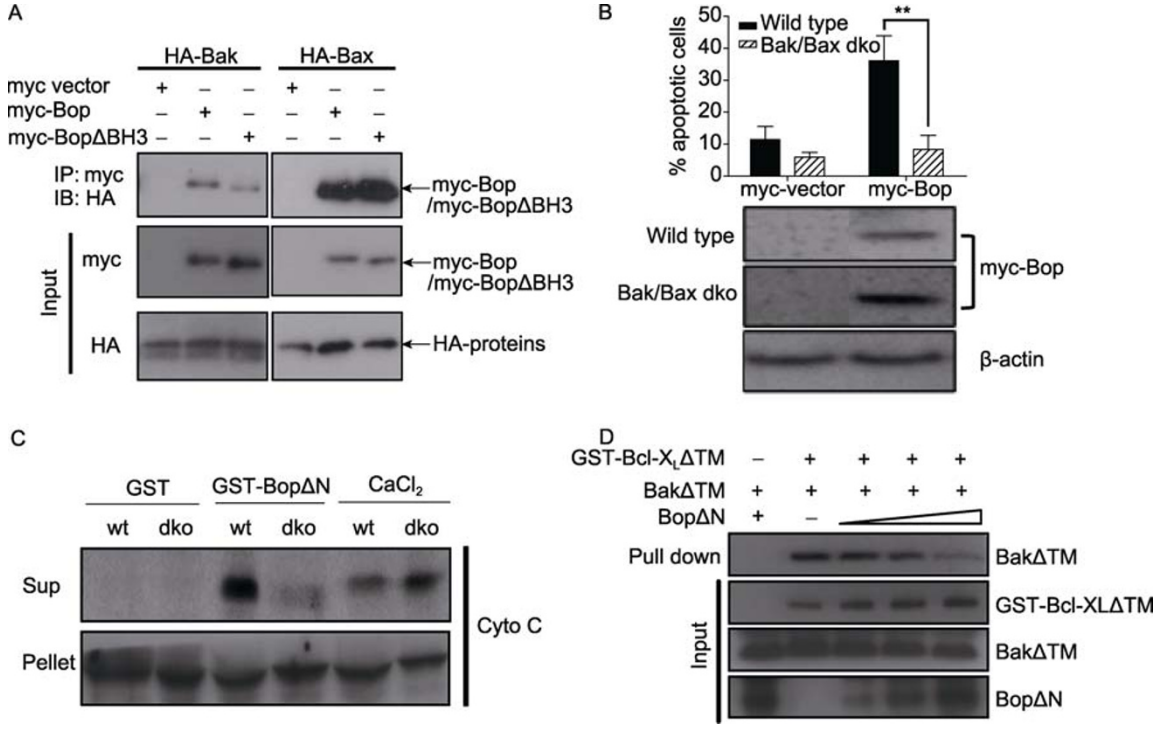

Figure 7. Bop functions primarily through Bax and Bak. (A) Intermolecular interactions between Bop and Bax or Bak were analyzed by co-immunoprecipitation of 293T cell lysates transfected with pCMV-myc-Bop and pCMV-HA-Bax, or pCMV-HA-Bak, in a similar condition to Fig. 5C. pCMV-myc-BopΔBH3 was also included in parallel experiments. The inputs of various proteins were analyzed by immunoblotting of cell lysates with antibody to myc or HA (bottom panels). (B) Apoptotic rates of wild type (wt) or Bak/Bax double knockout (Bak/Bax dko) MEF cells were analyzed after transfected with pCMV-myc-Bop and pDS-Red2 (0.1 μg) which was include as an indicator of transfected cells. About 10^4 red-emitting cells were gated and apoptotic cells were analyzed by Annexin V-FITC staining in flow cytometry 36 h after transfection (top). The expression levels of myc-Bop were measured by immunoblotting with β-actin as a loading control (bottom). The data are the average of three independent experiments (mean ± s.e.m.). (C) Cytochrome c release from wt or Bak/Bax dko mitochondria (50 μg) after incubation with GST-BopΔN (800 nmol/L) was analyzed as in Fig. 3C. GST (800 nmol/L) or $CaCl_2$ (200 μmol/L) was included as negative or positive control. (D) Displacement of Bak from Bcl-X_L by Bop was analyzed in a tripartite GST pulldown assay using an increasing amounts of BopΔN (250, 500, 1000 nmol/L) to challenge the pre-formed heterodimeric complex formed by a fixed amount of GST-Bcl-X_LΔTM (250 nmol/L) and recombinant BakΔTM (500 nmol/L). Association of Bak with Bcl-X_L was then assessed by immunoblotting with antibody to Bak (top). The input of each protein was immunoblotted with antibody to GST, Bak or Bop, respectively (bottom).

treatment to release cytochrome c (Fig. 7C). As a control, $CaCl_2$-induced cytochrome c release was not affected by the absence of Bax/Bak (Fig. 7C). BopΔN and Bop behaved similarly in terms of association with Bak and Bax (Fig. S5C). Finally, to test whether Bop can displace Bak from Bcl-X_L confinement to exert its pro-apoptotic activity, we further performed tripartite competition assays by mixing recombinant BakΔTM and GST-Bcl-X_LΔTM proteins with increasing amounts of BopΔN. GST pulldown showed that BakΔTM associated with GST-Bcl-X_LΔTM less efficiently in the presence of more BopΔN protein (Fig. 7D). Therefore, these results suggested that Bop could overcome Bcl-X_L restriction on Bak/Bax to trigger mitochondrial dysfunction and release of cytochrome c.

DISCUSSION

We demonstrated in this work that human Bop may be a novel BH3-only member of Bcl-2 protein family. Based on its properties of mitochondrial residence, modulating mitochondrial outer membrane permeability and interaction with Bcl-2 family proteins, we further propose that Bop may play a role in intrinsic apoptotic pathway.

All BH3-only proteins bind with high affinity to at least some pro-survival Bcl-2 members (van Delft and Huang, 2006). We find in this report that all five pro-survival Bcl-2 members contact and counteract Bop. Indeed, pro-survival Bcl-2 proteins seem to be necessary to detain an otherwise over-reactive Bop protein in resting cells. This is

because phosphorylated thereby inactivated Bcl-2 by VCR could potentially release Bop for initiation of downstream apoptotic cascade. Therefore, down-regulation of Bop gene expression renders cells resistance to VCR-induced cell death. That phosphorylated Bcl-2 fails to heterodimerize with Bop is reminiscent of previous findings that phosphorylated Bcl-2 dissociates from Bax (Srivastava et al., 1998; Poommipanit et al., 1999) or Beclin 1 (Liu et al., 2012). On the other hand, Bop may be a potential apoptotic inducer through antagonizing pro-survival Bcl-2 proteins. This can be explained by the finding that Bop can release Bak from Bcl-X_L constraint and trigger efficient cell killing and cytochrome c releasing in a Bak/Bax-dependent manner. Therefore, we postulate that, upon appropriate apoptotic stimuli, constrained Bop is released from phosphorylated Bcl-2 and induces cell death by liberate Bak or Bax to form cytochrome c permeable pores.

There are currently two main models of Bak and Bax activation, known as the "direct" or "displacement" model, albeit another "embedded together" model combining the two is also proposed (Giam et al., 2008; Leber et al., 2010; Shamas-Din et al., 2011). In both models, BH3-only proteins promote apoptosis by antagonizing pro-survival Bcl-2 proteins and releasing activator BH3-only proteins (such as Bid or Bim) or pro-apoptotic effectors (such as Bak or Bax) from sequestration, which will trigger oligomerization and activation of Bak/Bax for cytochrome c release. We therefore are tempted to postulate that Bop could activate Bak/Bax and induce cell death by direct protein interaction with Bcl-2 family members. However, whether Bop/Bcl-2 and Bak/Bax-Bcl-2 are in the same or different complexes, alternatively, whether Bop triggers apoptosis in single- or two-hit mode remains to be investigated.

Besides the potential mechanism that Bop induces apoptosis via interaction with Bcl-2 family members, Bop may adopt an alternative strategy to induce mitochondrial dysfunction. Previous studies have indicated that VDAC1 is a component of permeability transition pore (PTP) in the mitochondrial outer membrane and involved in release of apoptogenic factors, such as cytochrome c (Crompton, 1999; Crompton et al., 2002; Tsujimoto and Shimizu, 2002). Pro-apoptotic Bcl-2 proteins can interact with VDAC to form a protein-permeable pore in the out mitochondrial membrane (Shimizu et al., 1999; Shimizu et al., 2000; Sugiyama et al., 2002; Banerjee and Ghosh, 2004). Experiments with blocking antibodies against VDAC1 indicate that Bop can contact the loop regions of VDAC1. This raises the possibility that Bop has an ability to activate VDAC1 directly, like Bax (Shimizu et al., 2001) or Bim (Sugiyama et al., 2002). Furthermore, it remains intriguing to determine which permeability pore, Bak/Bax or VDACs, is utilized by Bop, considering that Bop contacts both Bak and VDAC1.

In conclusion, we have reported Bop, which is a new relative of BH3-only protein sub-family, may mediate mitochondria-dependent apoptogenic effect through both VDAC1 channel and Bak/Bax pores. The direct interaction network of Bcl-2 family members may play a key role in modulation of Bop activity.

MATERIALS AND METHODS

Reagents

The following antibodies were used: myc (clone 9E10), Mcl-1 (clone S-19), HA, GST and Bak (all rabbit polyclonal) from Santa Cruz Biotechnology (Santa Cruz, CA), Bcl-X_L (clone 54H6), phosphor-Bcl-2 (clone 5H2) and COX-IV (rabbit polyclonal) from Cell Signaling Technology (Danvers, MA), Bcl-2 (clone E17) from Epitomics (Burlingame, CA), GFP (rabbit polyclonal) from Abcam (Cambridge, UK), His (clone 3D5) from Invitrogen (Carlsbad, CA), VDAC1 (clone 31HL) from Calbiochem (Billerica, MA), β-actin (clone AC-15) from Sigma-Aldrich (St. Louis, MO), cytochrome c (clone 7H8.2C12) from Imgenex (San Diego, CA). All chemicals were from Sigma-Aldrich. Other reagents used were Glutathione Sepharose 4B from Amersham Biosciences (Waukesha, WI), Protein G Agarose from Millipore (Billerica, MA), complete protease inhibitors cocktail from Roche (Basel, Switzerland), Annexin V-Cy5 Apoptosis Detection Reagent from Abcam, Recombinant human TNF-α from R&D (Minneapolis, MN).

Plasmids and Bop polyclonal antibody

The cDNA of full length Bop (Open Biosystems) were sub-cloned by add-on PCR to pEGFP-N1 or pCMV-myc (Clontech). Point mutations of the conserved amino acids of Bop BH3 domain were produced by site-directed mutagenesis according to manufacturer's manual (QuikChange, Stratagene) with modification on primer designing (Taylor et al., 1985). All constructs were confirmed by DNA sequencing analysis. Various prokaryotic and eukaryotic expression vectors carrying tBid, Bax, Bak, Mcl-1, Bcl-X_L or human VDAC1 were mentioned in the previous report (Weng et al., 2005).

Bop polyclonal antibody was obtained by mixing the recombinant BopΔN (50 μg) with the complete Freund's adjuvant to immunize Balb/c mice intramuscularly. Booster immunizations were performed with the incomplete Freund's adjuvant twice at 2-week interval. The antigenic specificity was verified in western blotting against the recombinant BopΔN protein.

Cell Culture, transfection, and apoptosis Assay

HeLa, 293T, A431 and various MEF cells (Bak/Bax dko and wild type) were routinely cultured in Dulbecco's modified Eagle's high glucose medium (HyClone) containing 10% fetal bovine serum (GIBCO). Indicated plasmids were transiently transfected into HeLa, 293T or A431 cells by Lipofectamine 2000 (Invitrogen) or into MEF cells by jetPEI (Polyplus) according to manufacturer's manual.

To assess apoptotic rates, HeLa cells (~$1x10^6$) were transiently transfected with indicated plasmids. About 10^4 GFP positive cells were gated and apoptotic cells were counted by Annexin V-Cy5 and PI staining using flow cytometry (BD FACSCalibur) at the indicated

time. Apoptotic rates are the average from at least three independent experiments (mean ± s.e.m.).

GST pulldown and co-immunoprecipitation assays

Recombinant GST-BopΔN and its mutant derivative proteins, GST-tBid, GST-Mcl-1 and His-VDAC1 were purified and GST pulldown assays were performed as previously described (Shi et al., 2003; Weng et al., 2005). In brief, 5 μg His-VDAC proteins in 0.5 mL binding buffer [20 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Nonidet P-40] were incubated with recombinant GST or GST-tagged proteins (5 μg) pre-immobilized on glutathione beads at 4°C for 1 h. The pulled-down proteins were separated in 12% SDS- PAGE and immunoblotted with His-tag antibody. Where indicated, 1 μg antibody to N-terminus (31HL), loops (#20, #25) or C-terminus (#30) of VDAC1 was pre-incubated with the recombinant His-VDAC1 as previously described (Shi et al., 2003; Weng et al., 2005).

Tripartite complex GST pulldown assays were performed as described previously (Shi et al., 2003; Weng et al., 2005). In brief, 250 nmol/L GST-Bcl-X_LΔTM was incubated with 500 nmol/L recombinant BakΔTM proteins in 200 μL pulldown buffer (20 mmol/L Hepes, pH 7.4, 150 mmol/L KCl, 1mmol/L EDTA, and 0.5% NP-40) at 4°C for 1 h. Increasing amounts of recombinant BopΔN (250, 500, 1000 nmol/L) were then added to the preformed Bak/Bcl-X_L complex for additional 1 h. Glutathione Sepharose 4B beads (20 μL) were then added to the mixture and rocked gently at 4°C for 1 h. Beads were extensively washed with the same pulldown buffer before the proteins were eluted and separated in 12% SDS–PAGE. The amounts of BakΔTM pulled down by GST-Bcl-X_LΔTM were assessed by western blotting using anti-Bak antibody. The input of each protein was quantified by immunoblotting with anti-GST, -Bak and -Bop antibodies, respectively.

For co-immunoprecipitation experiments, 293T or HeLa cells (1 x 10^6) transfected with indicated plasmids were lysed in 350 μL IP buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1mmol/L PMSF, 0.5% Nonidet P-40 and protease inhibitor cocktail) at 48 h after transfection, and 300 μL lysates of which were immunoprecipitated with 5 μg/mL of HA antibody at 4°C overnight. Immunocomplexes were then absorbed by 5% (*v/v*) of protein G Agarose (Millipore, USA) for an additional hour before separated in 12% SDS–PAGE. The desired proteins and expression levels of each protein (50 μL whole cell lysates) were determined by western blotting with corresponding antibodies.

Cytochrome c release and mitochondrial permeability transition

Balb/c mice liver mitochondria were isolated and cytochrome c release experiments (15 μg mitochondria proteins) were performed essentially as described previously (Weng et al., 2005). Mitochondrial membrane potential (ΔΨm) was assessed as described in reference (Zheng et al., 2004). Briefly, isolated mitochondria (0.1 mg protein/mL) were loaded with 30 nmol/L Rhodamine 123 and incubated at 25°C in the PT-2 medium (250 mmol/L sucrose, 2 mmol/L HEPES, pH 7.4, 0.5 mmol/L KH_2PO_4 and 4.2 mmol/L potassium succinate) to energize mitochondria. GST, GST-BopΔN or GST-Bcl-X_L (150 μg) was added at indicated time. ΔΨm was assessed by measuring the ΔΨm dependent release of Rhodamine 123 from mitochondria using a spectrofluorimeter (Jobin-Yvon FluoroMax-2). As a control, mitochondrial depolarization was induced by addition of 50 μmol/L protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) at the end of the experiments. Mitochondrial swelling was monitored by the decrease of 90° light scatter as described in reference (Zheng et al., 2004). In brief, isolated mitochondria (0.1 mg protein/mL) were incubated at 25°C in PT-2 medium. Mitochondrial swelling was monitored by the decrease of 90° light scatter at λ = 520 nm using a Jobin-Yvon FluoroMax-2 spectrofluorimeter. 100 μmol/L $CaCl_2$ was added at the end of experiments as a positive control for permeability transition pore opening.

Assay of protein localization by confocal laser scanning microscopy

A431 cells were transiently transfected with indicated expression plasmids for GFP or GFP-tagged proteins. 24 h after transfection, cells were plated at 10%–20% confluency on glass coverslips in 35 cm culture plates. After another 24 hours, cells were incubated with MitoTracker Red (50 nmol/L) for 30 min at 37°C. Cells were then washed and fixed in 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. Finally, coverslips were washed three times with PBS and mounted onto microscope slides for confocal microscopy (Olympus LSCMFV1000).

For immunofluorescence staining of endogenous Bop, Hela cells plated on glass were fixed and permeabilized in the same condition as above. Cells were then blocked with 5% BSA in PBS for 1 h at room temperature. After incubating with primary antisera and FITC-labeled secondary antibody, coverslips were washed with PBS and mounted onto microscope slides for confocal microscopy.

ImageStream analysis

A431 cells (1 x 10^6) expressing GFP or GFP-tagged proteins (Bop and Bcl-2) were labeled with MitoTracker Red and then suspended in 100 μL PBS for imaging flow cytometry (ImageStream 100, Amnis, Seattle). At least 20,000 events/sample were collected and data were analyzed using IDEAS image analysis software. In brief, single, focused, GFP-MitoTracker double positive cells were identified by gating on cells with high aspect ratios (minor axis divided by major axis, eliminating conjugates and debris), and high contrast (measured by the Gradient RMS Feature). Degree of co-localization was analyzed using bright detail similarity algorithm, a pixel-by-pixel and cell-by-cell comparison of two images captured by GFP (GFP-tagged proteins) and Mito (MitoTracker Red) channels. Using co-localization wizard provided by IDEAS, co-localized cells in the SSC (side scatter) and Mito channels were used to first determine threshold similarity value. Gaussian distributions of bright detail similarity (SSC vs Mito) were generated and the size of positive region (High Sim) was adjusted so that 1% cells fell within this area (cutoff = 1.83, representing 99% confidence that the two images positively correlated). This algorithm was then applied to quantitate Bop and Bcl-2 colocalization with mitochondria.

Reduction of endogenous Bop expression in cells

The vectors of pGPH1-GFP-shRNA expressing both GFP and shRNA targeting human Bop or scrambled shRNA control (purchased from GenePharma, Shanghai) were transfected in HeLa cells as indicated. Transfectants were either harvested for Bop mRNA and protein analysis, or treated with various apoptotic stimuli 72 h after transfection. Target sequences for shRNA for Bop knockdown were as following: Bop shRNA-1: 5′-GGCAATACTTAGC TAGGTTCT-3′; Bop shRNA-2: 5′-GACTGTCGGAGTCAGCTAA TC-3′ and scrambled-shRNA: 5′-GTTCTCCGAACGTGTCACGT-3′.

Semi-quantitative RT-PCR analysis

HeLa cells were harvested and total RNA was isolated using TRIzol reagent (Invitrogen) closely following the supplier's recommendation. 2 μg of total RNA was annealed with oligo$(dT)_{12–18}$ and reversed-transcribed using M-MLV (Moloney murine leukemia virus) reverse transcriptase (Invitrogen) for the synthesis of cDNA. The GAPDH and Bop mRNAs were amplified for 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s on a 7500 Real-Time PCR System (Applied Biosystems). For specific detection of Bop, two primers were as follows: 5′-GTCAGCAGGGCCCTCGCATTC-3′ (forward) and 5′-ACCCCTTTCTGCAGGTTTACC-3′ (reverse).

Northern blotting

Total RNA (20 μg) was isolated from indicated human tissues according to manufacturer's manual (ULTRAhyb, Ambion) and Northern blotting was performed using a Bop cDNA probe according to manufacturer's manual (Rediprime II DNA Labeling System, Amersham Biosciences).

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