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Identification of a Motif in BMRP Required for Interaction With Bcl-2 by Site-Directed Mutagenesis Studies

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ABSTRACT

Bcl-2 is an anti-apoptotic protein that inhibits apoptosis elicited by multiple stimuli in a large variety of cell types. BMRP (also known as MRPL41) was identified as a Bcl-2 binding protein and shown to promote apoptosis. Previous studies indicated that the amino-terminal two-thirds of BMRP contain the domain(s) required for its interaction with Bcl-2, and that this region of the protein is responsible for the majority of the apoptosis-inducing activity of BMRP. We have performed site-directed mutagenesis analyses to further characterize the BMRP/Bcl-2 interaction and the pro-apoptotic activity of BMRP. The results obtained indicate that the 13–17 amino acid region of BMRP is necessary for its binding to Bcl-2. Further mutagenesis of this motif shows that amino acid residue aspartic acid (D) 16 of BMRP is essential for the BMRP/Bcl-2 interaction. Functional analyses conducted in mammalian cells with BMRP site-directed mutants BMRP(13Ala17) and BMRP(D16A) indicate that these mutants induce apoptosis through a caspase-mediated pathway, and that they kill cells slightly more potently than wild-type BMRP. Bcl-2 is still able to counteract BMRP(D16A)-induced cell death significantly, but not as completely as when tested against wild-type BMRP. These results suggest that the apoptosis-inducing ability of wild-type BMRP is blocked by Bcl-2 through several mechanisms. J. Cell. Biochem. 113: 3498–3508, 2012.

KEY WORDS: BCL-2; BMRP; APOPTOSIS; MITOCHONDRIA; RIBOSOME

Cell death by apoptosis is required for the normal development of metazoans, as well as for the maintenance of tissue homeostasis and normal functioning of the adult organism [Raff, 1992; Jacobson et al., 1997; Danial and Korsmeyer, 2004]. Apoptosis is characterized by specific morphological features, which include cell shrinkage, condensation of the nuclear chromatin, plasma membrane blebbing, and fragmentation of the cell into smaller pieces (apoptotic bodies) that end up being phagocytosed by macrophages and other neighboring cells. Abnormal levels of apoptosis have been implicated in the etiology of human diseases including cancer, disorders of the immune system, infertility, neurodegenerative diseases (such as Alzheimer's, and Huntington's disease), as well as cardiac arrest and stroke damage [Vaux et al., 1988; Barinaga, 1998ab; Mattson, 2000; Green and Evan, 2002; Rathmell and Thompson, 2002; Danial and Korsmeyer, 2004; Fesik, 2005; Adams and Cory, 2007]. In mammals, this orderly and efficient form of physiological cell death is regulated and executed by a diverse set of protein families [Strasser et al., 2000; Danial and Korsmeyer, 2004]. The members of these families perform their functions as part of two main and distinct mammalian apoptosis signaling pathways that, however, converge in their final stages, with cross-talk also occurring between the two pathways. These two

Abbreviations: Ab, antibody; AD, activation domain; Ala, alanine; BD, binding domain; BH domain, Bcl-2 homology domain; BMRP, Bcl-2 interacting mitochondrial ribosomal protein L41; C, carboxyl; D, aspartic acid; ER, endoplasmic reticulum; GFPnuc, nuclear AcGFP1; His, histidine; HRP, horseradish peroxidase; IP3R, inositol 1,4,5-triphosphate receptor; Leu, leucine; MOMP, mitochondrial outer membrane permeabilization; N, amino; ORF, open reading frame; PCR, polymerase chain reaction; R, arginine; SD, synthetic defined; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TM, transmembrane; Trp, tryptophan; WB, Western blot; WT, wild-type.

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pathways are known as the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway [Strasser et al., 2000; Danial and Korsmeyer, 2004]. The mitochondrion acts as a prominent death regulator in the intrinsic apoptosis pathway, but it is also involved in the extrinsic pathway. Among the proteins that participate in this form of cellular suicide, the Bcl-2 family of proteins plays an important role in the regulation of apoptosis [Adams and Cory, 2001; Youle and Strasser, 2008]. This family comprises more than 20 members, some of which protect cells from apoptosis while others promote cell death. Bcl-2 family members control the stability of the mitochondrion, thereby regulating the release of mitochondrial apoptogenic factors into the cytosol, where these factors are required for caspase activation and, therefore, cell death by apoptosis [Kluck et al., 1997; Adams and Cory, 2001; Lucken-Ardjomande and Martinou, 2005; Youle and Strasser, 2008].

Bcl-2 is an anti-apoptotic protein that has been shown to protect cells from death induced by a large number of apoptotic signals [Vaux et al., 1988; Zhong et al., 1993; Reed, 1994]. The detailed mechanisms by which Bcl-2 promotes survival have not been fully elucidated. To gain further insight into the mechanisms of action and regulation of Bcl-2, we sought to identify additional proteins that bind to Bcl-2 using a yeast Two-Hybrid approach. These studies identified BMRP (Bcl-2 interacting mitochondrial ribosomal protein L41, also known as MRPL41) as a novel Bcl-2 binding protein [Chintharlapalli et al., 2005]. BMRP overexpression induces cell death in a variety of primary and immortalized mammalian cell lines. This cell death exhibits the characteristic morphological features of apoptosis, and is counteracted by Bcl-2 as well as p35, a broad-spectrum caspase inhibitor [Chintharlapalli et al., 2005; Malladi et al., 2011]. Deletion mutational analyses showed that the 4 BH (Bcl-2 homology) domains of Bcl-2, BH1 through BH4, are required for the BMRP/Bcl-2 interaction. These deletion studies also delimited the region of BMRP needed for its interaction with Bcl-2 to the amino (N)-terminal two-thirds of the BMRP protein, and they indicated that the cell death-inducing domains of the protein also reside mainly within its N-terminal two-thirds [Malladi et al., 2011]. Additional reports have shown BMRP to be involved in p53-mediated apoptosis that is elicited by growth-inhibitory conditions, such as serum deprivation and treatment with actinomycin D. In these studies, BMRP was reported to increase the stability of the p53 protein and its translocation to the mitochondria, thereby inducing p53-dependent apoptosis (in a transcription-independent fashion) [Yoo et al., 2005]. BMRP has also been shown to induce cell cycle arrest in the absence of p53 [Kim et al., 2005].

The present study reports the results of site-directed mutagenesis analyses performed with the BMRP protein to further characterize the BMRP/Bcl-2 interaction and the pro-apoptotic activity of BMRP. The results of these studies indicate that the amino acid region 13–17 of BMRP is required for its binding to Bcl-2. Within this motif, amino acid residue aspartic acid (D) 16 of BMRP is essential for the BMRP/Bcl-2 interaction. Functional studies show that the BMRP site-directed mutants BMRP(13Ala17) and BMRP(D16A) induce apoptosis, therefore suggesting that BMRP does not promote apoptosis by interacting with and blocking the anti-apoptotic activity of Bcl-2. A more likely possibility is that the apoptosis-inducing ability of BMRP is counteracted by Bcl-2 at various levels.

MATERIALS AND METHODS

ALIGNMENT OF BMRP PROTEIN SEQUENCES

The protein sequence corresponding to human BMRP (hBMRP) was aligned with the BMRP sequences of eight other species obtained from the GenBank. The GenBank accession numbers of the sequences utilized for the alignment are as follows: *Mus musculus* BMRP, NP_001026978 (GI: 113461980); *Xenopus laevis* BMRP, NP_001086478 (GI: 1478995330); *Danio rerio* BMRP, NP_001018603 (GI: 66472774); *Strongylocentrotus purpuratus* BMRP, XP_785814 (GI: 72008280); *Drosophila melanogaster* BMRP, NP_611022 (GI: 21687222); *Caenorhabditis elegans* BMRP, NP_493695 (GI: 17531321); *Arabidopsis thaliana* BMRP, NP_198824 (GI: 15242565); and *Saccharomyces cerevisiae* BMRP, AAB21096 (GI: 7550153). The alignment was performed by the Clustal method.

PLASMID CONSTRUCTION AND SEQUENCING

For interaction studies, a set of six hBMRP alanine (Ala) substitution mutants (BMRP(13–17), BMRP(22–25), BMRP(34–39), BMRP(54–59), BMRP(71–76), and BMRP(83–87)) was generated by site-directed mutagenesis utilizing standard polymerase chain reaction (PCR) protocols [Innis et al., 1990]. Specific oligonucleotide primers were designed for the purpose (all primers used in this study were obtained from Sigma–Aldrich Inc.), and the plasmid pcDNA3-hbmrp [Chintharlapalli et al., 2005] was utilized as template in these PCR reactions. The amplified DNA fragments were digested with *Bam*HI and *Eco*RI, and subcloned into the pBluescript-KS plasmid following standard molecular biology protocols [Sambrook and Russell, 2001]. The amplified DNA fragments were sequenced using the dideoxynucleotide chain-termination method (utilizing the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems). All fragments were then subcloned into the yeast Two-Hybrid vector pGAD-GH (Clontech) in frame with the Gal4 activation domain (Gal4AD) coding region utilizing the *Bam*HI and *Eco*RI restriction sites. A second set of hBMRP site-directed mutants was constructed for yeast Two-Hybrid studies (BMRP(R13A), BMRP(D16A), and BMRP(R17A)) using the same strategy described above. Plasmids pGBT8-hbcl2Δ13, pGAD10-hbad and pGAD-GH-hbmrp have been previously described [Chintharlapalli et al., 2005; Malladi et al., 2011].

For expression studies in mammalian cells, the plasmids pcDNA3-hbmrp(13Ala17) and pcDNA3-hbmrp(D16A) were generated, which drive the expression of the BMRP Ala substitution mutants BMRP(13Ala17) and BMRP(D16A), respectively. The cloning procedures utilized to generate these additional constructs were essentially as described above. The plasmids pSFFVNeo-hbcl-2 and pcDNA3-p35, as well as the reporter plasmid pcDNA3-β-galactosidase, used in functional studies, have been described elsewhere [Chintharlapalli et al., 2005]. The cell line NIH/3T3 (obtained from ATCC, CRL-1658) was utilized in cell death and morphological apoptosis studies because NIH/3T3 cells die by apoptosis when treated with a variety of apoptotic stimuli [Dorstyn and Kumar, 1997; Schenning et al., 2004]. HEK 293T (Human Embryonic Kidney 293T) cells were used for expression studies since they are oncogenically transformed cells and, thereby, capable of

overexpressing high levels of pro-apoptotic proteins without undergoing apoptosis (HEK 293T cells are significantly more resistant to apoptosis than NIH/3T3 cells).

YEAST COTRANSFORMATION AND DROP ASSAY

Several plasmid combinations were cotransformed into the yeast strain HF7c using a yeast transformation kit (Sigma). A drop assay was carried out as previously described [Chintharlapalli et al., 2005; Malladi et al., 2011] to assess the ability of the various yeast cell cotransformants to grow in a medium lacking histidine (His).

YEAST PROTEIN EXTRACT PREPARATION AND WESTERN BLOT ANALYSIS

Protein extracts from yeast pellets were prepared and equivalent amounts of lysates were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose, and immunoblotted with a 1:1,000 dilution of an anti-Gal4AD polyclonal antibody (Ab) (affinity purified rabbit Ab; Sigma) as described [Malladi et al., 2011]. Prior to immunoblotting, the membrane was reversibly stained with Ponceau S to ensure equal sample loading and protein transfer. Additionally, equal loading was confirmed by immunoblotting with a 1:4,000 dilution of mouse anti-α-tubulin Ab (Sigma).

CELL DEATH ASSAY

NIH/3T3 cells were cultured for transfection in multiwell-12 tissue culture plates (approximately 4×10^4 cells/well). Fourteen hours after seeding, cells were transfected with the indicated plasmids (400 or 900 ng/well) along with a smaller amount of pcDNA3-β-galactosidase as a reporter (200 ng/well). Transfections were performed by lipofection (Lipofectamine™ 2000; Invitrogen) following the manufacturer's recommendations. When necessary, empty plasmid was added so that the total amount of DNA per well was always equivalent (1 μg/well total was used for the experiments shown in Fig. 4A,B, and 1.5 μg/well total was utilized for the assays presented in Fig. 4E,F). Survival of transfected cells was measured by quantification of reporter gene activity (β-galactosidase activity) 48 h post-transfection as described previously [Chintharlapalli et al., 2005; Malladi et al., 2011]. A value of 100% survival was assigned to the β-galactosidase activity of cells transfected with empty vector, and the percentage cell survival was calculated for each one of the samples. Transfections with only pcDNA3 plasmid (without the pcDNA3-β-galactosidase reporter plasmid) were used to set the 0% β-galactosidase baseline (providing the 0% survival mark for the other transfections). Statistical analyses were conducted as previously described [Malladi et al., 2011]. Briefly, two-tailed Student *t*-tests were performed to compare the results obtained for each condition tested (overexpression of the protein or proteins of interest) with the results obtained with the control (β-gal) for each assay. The averages of the two samples (experimental condition vs. the control) were considered statistically different whenever P was lower than 0.01.

TRANSFECTION OF HEK 293T CELLS AND WESTERN BLOT ANALYSIS OF HEK 293T LYSATES

HEK 293T cells were seeded in multiwell-6 tissue culture plates (approximately 4×10^5 cells/plate) for transient transfection (performed 12 h after seeding) with eukaryotic expression vectors (4 μg) encoding wild-type (WT) BMRP, BMRP(13Ala17), or BMRP(D16A). A negative control was transfected with empty vector. Transfection was carried out by lipofection using Lipofectamine™ 2000 reagent (Invitrogen). Approximately 48 h after transfection, cells were rinsed once with PBS [Sambrook and Russell, 2001] and frozen (−80°C). Lysates were prepared in 250 μl of 2X STT-PIC buffer [Chintharlapalli et al., 2005]. Equivalent amounts of cell lysates were resolved on a SDS-12% polyacrylamide gel. Immunobloting was carried out with a 1:1,000 dilution of rabbit polyclonal anti-BMRP serum (generated in our laboratory, [Chintharlapalli et al., 2005]), followed by incubation with a 1:5,000 dilution of goat anti-rabbit-HRP Ab (Pierce). Equal loading was confirmed by reversible staining of the nitrocellulose membrane with Ponceau S after protein transfer, and Western blotting with mouse anti-α-tubulin Ab (Sigma).

MORPHOLOGICAL APOPTOSIS ASSAY

NIH/3T3 cells were transiently transfected by lipofection essentially as described [Malladi et al., 2011]. Briefly, cells seeded in multiwell-12 tissue culture plates (approximately 2.5×10^4 cells/well) were transfected 20 h after seeding with the indicated plasmids (800 ng/well; pcDNA3-AU1-mbad, pcDNA3-hbmrp, pcDNA3-hbmrp(13Ala17), or pcDNA3-hbmrp(D16A), which drive the expression of murine (m) Bad, hBMRP, hBMRP(13Ala17), or hBMRP(D16A), respectively), and 200 ng/well of pAcGFP1-Nuc (Clontech), which encodes nuclear AcGFP1. The coexpression approach allows the identification of the cells that are successfully transfected, and thus are exposed to the apoptotic stimulus, from the untransfected cells (Fig. S2). Empty pcDNA3 plasmid was used for the control so that the total amount of DNA per well was also 1 μg. Transfected cells were examined (from 16 h through 3 days post-transfection) under a phase contrast Olympus-CK40 microscope equipped with fluorescence capabilities, and photomicrographs were taken with a Pixera Penguin 150 CL camera. For simplicity, the m prefix will not be used when referring to the mBad protein in other sections of this article.

For quantitation of cell death caused by BMRP overexpression, cotransfection experiments were performed with pAcGFP1-Nuc plasmid, and pcDNA3-hbmrp or control pcDNA3 plasmid, as indicated above. Images of three specific fields were taken under the fluorescence microscope at 40 and 60 h following transfection, and the number of nuclear AcGFP1 (GFPnuc) positive cells in each field was quantitated utilizing Image Processing Tool Kit 4.0 imaging analysis software (Reindeer Graphics). The images were initially converted to grayscale, and then subjected to a threshold filter using Adobe Photoshop software, to analyze cells with high levels of expression. The average ± SD number of GFPnuc positive cells was calculated for the control and BMRP cotransfections, and then normalized to the control transfection to estimate the reduction in cell numbers caused by BMRP coexpression. Statistical analysis was performed essentially as described above. Results were considered statistically significant when $P < 0.05$.

RESULTS

IDENTIFICATION OF BMRP MOTIFS THAT ARE REQUIRED FOR ITS INTERACTION WITH Bcl-2

Deletion mutational analyses and binding studies performed with BMRP showed that the carboxyl (C)-terminal third of the BMRP protein is dispensable for the BMRP/Bcl-2 interaction (BMRP is a 137-amino acid residue-long protein), while deletion of the N-terminal third of the BMRP protein (first 45 amino acid residues) resulted in lack of binding to Bcl-2 [Malladi et al., 2011]. Smaller deletions (15 amino acid residues) on either side of the BMRP(1–92) mutant protein led to loss of binding to Bcl-2, probably because of effects on the tertiary structure of the protein [Malladi et al., 2011]. Therefore, to further characterize the regions of BMRP that are required for its interaction with Bcl-2, a substitution mutational approach was undertaken in which several small stretches of amino acids within the N-terminal two-thirds of BMRP were mutated to Ala. These substitution mutants were then utilized in binding studies using the yeast Two-Hybrid assay. Six regions within the 1–92 domain of BMRP were selected for site-directed mutagenesis based on the results of alignment studies performed with hBMRP and the BMRP proteins of other species (Fig. 1). Within these six homologous regions that were chosen for Ala substitution analyses, specific amino acid residues were selected for mutation based on the presumed potential of the side chain for participation in binding. Glycine and proline residues were left unchanged to prevent possible alterations in protein structure. A few other residues not highly conserved were not modified. A schematic representation of the six BMRP site-directed mutants generated is presented in Figure 2A. The DNA fragments encoding these six BMRP site-directed mutants were subcloned into the pGAD-GH yeast Two-Hybrid vector in frame with the Gal4AD coding region. The ability of Gal4AD/mutant BMRP hybrid proteins to interact with the Gal4BD(binding domain)/Bcl-2Δ13 hybrid protein was assessed by performing yeast cotransformations and Two-Hybrid drop assays [Malladi et al., 2011]. The Bcl-2Δ13 mutant was used for these binding studies since it lacks the transmembrane domain (TM) of Bcl-2 (amino acid residues 219–237 of Bcl-2) [Hanada et al., 1995; Hunter et al., 1996], which mediates the attachment of Bcl-2 to several cellular membranes, including the outer mitochondrial membrane, the nuclear envelope (outer membrane), and the endoplasmic reticulum (ER) membrane [Monaghan et al., 1992; Krajewski et al., 1993; Akao et al., 1994]. Since the Bcl-2Δ13 mutant is not retained to cytoplasmic membranes, it showed strong interaction with WT BMRP in Two-Hybrid drop assays [Malladi et al., 2011]. The results obtained in these assays (Fig. 2B) suggest that the amino acid region 13–17 of BMRP is required for the BMRP/Bcl-2 interaction, while the other motifs tested (22–25, 34–39, 54–59, 71–76, and 83–87) do

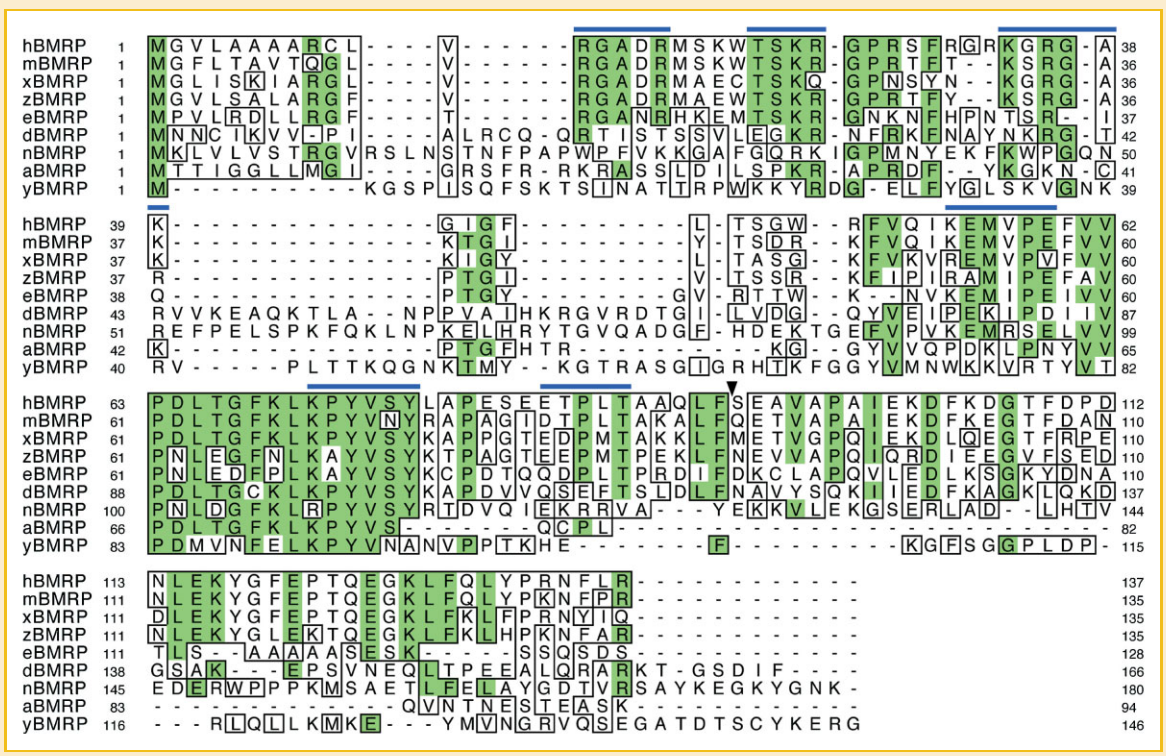

Fig. 1. Alignment of BMRP protein sequences. The sequence of the human BMRP (hBMRP) protein (top) was aligned with the BMRP sequences of 8 other species using the Clustal method. Sequence homology is indicated with the boxes, and sequence identity is shown by shading. The sequence to the right of the arrowhead was removed in the hBMRP deletion mutant BMRP(1–92). The blue lines on top of the aligned sequences indicate the amino acid stretches within the N-terminal two-thirds of the hBMRP protein that were targeted for mutation in the Ala substitution mutants. mBMRP, *Mus musculus* BMRP; xBMRP, *Xenopus laevis* BMRP; zBMRP, *Danio rerio* BMRP; eBMRP, *Strongylocentrotus purpuratus* BMRP; dBMRP, *Drosophila melanogaster* BMRP; nBMRP, *Caenorhabditis elegans* BMRP; aBMRP, *Arabidopsis thaliana* BMRP; yBMRP, *Saccharomyces cerevisiae* BMRP. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

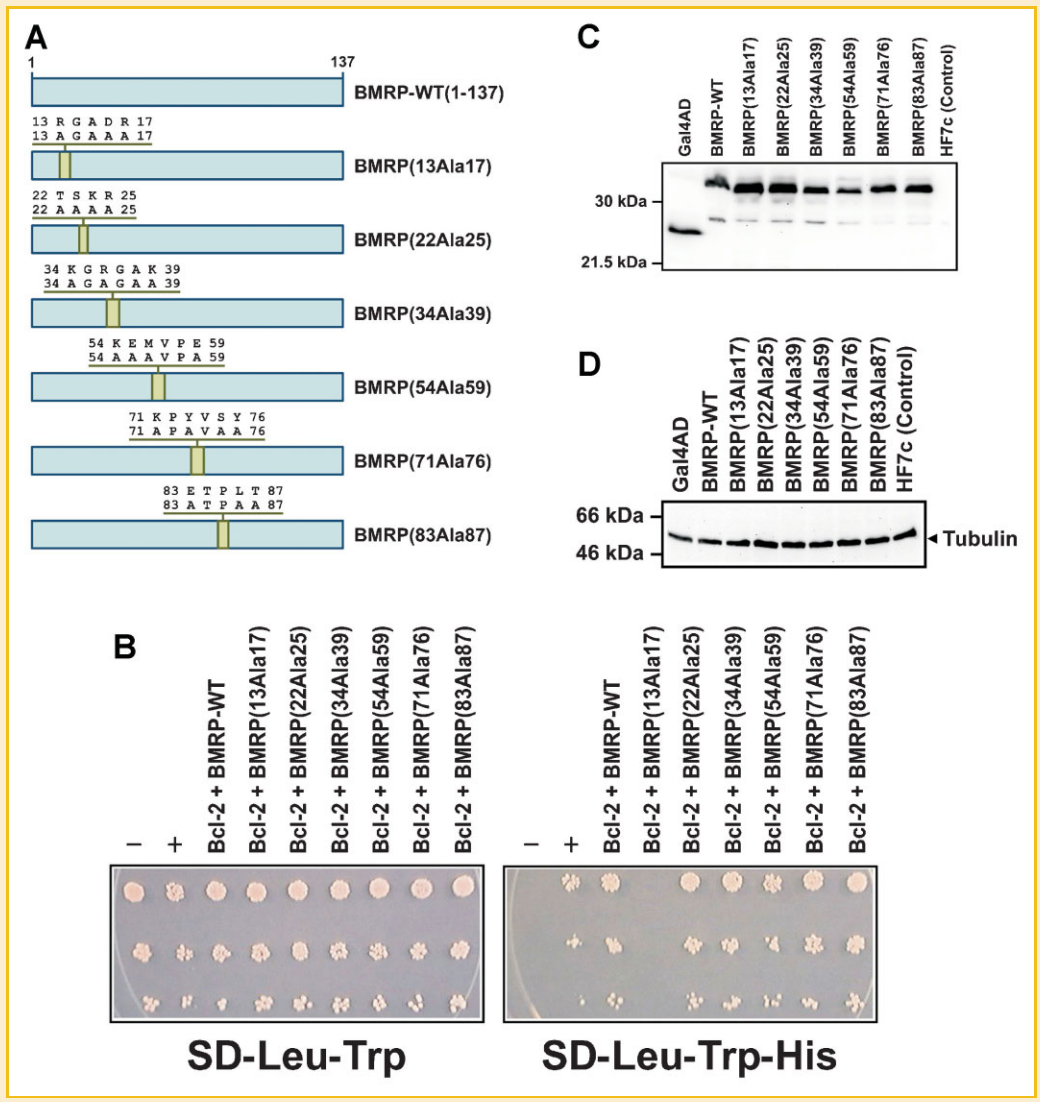

Fig. 2. Analysis of the interaction of BMRP site-directed mutants with Bcl-2 in yeast cells. A: A schematic representation of the six BMRP site-directed mutants generated is depicted. The specific amino acid residues that were replaced by Ala in each mutant protein are indicated on top of the corresponding diagram by showing the original and modified amino acid sequence of the motif. WT BMRP is also shown for comparison. WT BMRP is a 137-amino acid residue-long protein. B: Yeast Two-Hybrid assay performed using BMRP or its site-directed mutants fused to Gal4AD, and Bcl-2Δ13 fused to Gal4BD. The yeast strain HF7c was cotransformed with yeast Two-Hybrid vectors encoding BMRP or its site-directed mutants (BMRP(13–17), BMRP(22–25), BMRP(34–39), BMRP(54–59), BMRP(71–76), or BMRP(83–87)), and Bcl-2Δ13. The cotransformants were selected on synthetic defined agar medium lacking leucine and tryptophan (SD-Leu-Trp). A single yeast colony from each of the cotransformants was resuspended in sterile dH_2O and utilized to prepare three normalized serial dilutions. Five microliters from each of the three suspensions were spotted on SD-Leu-Trp and SD-Leu-Trp-His plates (SD agar medium lacking Leu, Trp, and His). Interaction between the proteins was assayed qualitatively by assessing the growth of HF7c cotransformed cells on SD-Leu-Trp-His (absence of growth is indicative of lack of interaction). (−), Negative control: HF7c cells cotransformed with the plasmid encoding the Gal4DBD/Bcl-2Δ13 fusion protein and the empty pGAD-GH plasmid. (+), The interaction between Bcl-2Δ13 and Bad proteins was utilized as a positive control in this assay. Note that the BMRP(13Ala17) mutant is unable to interact with Bcl-2Δ13, suggesting that the 13–17 amino acid region of BMRP is required for the BMRP/Bcl-2 interaction. The data presented is representative of at least four different colonies tested for each cotransformant. C: WB analysis of the expression of Gal4AD/BMRP site-directed mutants in yeast cells. Protein extracts from HF7c cotransformed cells expressing Gal4AD (negative control), Gal4AD/WTBMRP (positive control), or Gal4AD/BMRP site-directed mutants, were prepared and run on a SDS-12% polyacrylamide gel. Immunoblotting utilizing rabbit anti-Gal4AD serum demonstrated the expression of all BMRP Ala substitution mutant hybrid proteins. The predicted molecular masses of the proteins are as follows: Gal4AD, 21 kDa; Gal4AD/WTBMRP (or its site-directed mutants), 31 kDa. Please note that Gal4AD migrates slower than its predicted molecular mass, probably due to the acidic nature of the protein. Results are representative of three independent experiments. D: The lysates immunoblotted with the anti-Gal4AD Ab in part (C) were also immunoblotted with mouse anti-α-tubulin Ab to confirm equal loading. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

not seem necessary for this interaction (lack of interaction was detected only for the BMRP(13–17) mutant by the absence of growth on medium lacking His; please see Fig. 2B).

Expression of the various BMRP site-directed mutants in yeast cells was assessed by Western blotting. This analysis shows that all Gal4AD/mutant BMRP hybrid proteins were expressed at approximately similar levels (Fig. 2C). Equivalent loading of the yeast extracts utilized in the Western blot (WB) analysis was confirmed by reversible Ponceau S staining of the nitrocellulose membrane prior to immunblotting (data not shown), and by immunoblotting with an anti-α-tubulin Ab (Fig. 2D). Therefore, the lack of binding of the BMRP(13Ala17) substitution mutant to Bcl-2 does not seem to be due to the lack of expression of this mutant in yeast cells (please note that BMRP(13Ala17) expresses at the same or slightly higher levels than the other 5 Ala substitution mutants, and that this mutant is the only one that does not show interaction with Bcl-2 in yeast drop assays).

IDENTIFICATION OF INDIVIDUAL AMINO ACID RESIDUES OF BMRP THAT ARE ESSENTIAL FOR ITS INTERACTION WITH Bcl-2

The BMRP(13Ala17) mutant contains 3 Ala residues that replace amino acid residues arginine 13 (R13), aspartate 16 (D16), and arginine 17 (R17) of WT BMRP. Three additional BMRP site-directed mutants were generated that contain only one of the three mutations previously indicated (Fig. 3A), and the ability of these mutants to bind to Bcl-2 was also tested by conducting yeast Two-Hybrid assays. The data obtained shows that while mutants BMRP(R13A) and BMRP(R17A) are able to interact with Bcl-2, mutant BMRP(D16A) is not, suggesting that amino acid residue D16 of WT BMRP plays a critical role in the BMRP/Bcl-2 interaction (Fig. 3B; yeast cotransformants coexpressing Gal4BD/Bcl-2Δ13 and Gal4AD/BMRP(D16A) do not grow on medium lacking His, suggesting lack of interaction between Bcl-2Δ13 and BMRP(D16A)). The lack of binding between Gal4BD/Bcl-2Δ13 and Gal4AD/BMRP(D16A) does not seem to be due to lack of expression of the Gal4AD/BMRP(D16A) hybrid protein in yeast cells, since the three Gal4AD/mutant BMRP hybrid proteins were expressed at similar levels in the yeast protein extracts (Fig. 3C; equal loading of the WB shown in this figure was confirmed as previously mentioned for Fig. 2C; the corresponding anti-α-tubulin WB is shown in Fig. 3D).

FUNCTIONAL ANALYSIS OF BMRP(13Ala17) AND BMRP(D16A) SITE-DIRECTED MUTANTS

The WT BMRP protein binds to the anti-apoptotic protein Bcl-2, and it exhibits pro-apoptotic activity. One possible mechanism by which BMRP could exert is pro-apoptotic function is by binding to Bcl-2 and blocking the anti-apoptotic activity of Bcl-2. To test this hypothesis, the ability of the BMRP site-directed mutants BMRP(13Ala17) and BMRP(D16A) to induce apoptosis was assessed in NIH/3T3 cells. The open reading frames (ORFs) encoding the substitution mutants BMRP(13Ala17) and BMRP(D16A) were subcloned into a eukaryotic expression vector, and the corresponding constructs were transiently transfected into NIH/3T3 cells. Cell viability reduction assays performed with these cells indicate that both mutants induce cell death (Fig. 4A,B). In fact, the survival levels observed in cells overexpressing BMRP(13Ala17) and BMRP(D16A) were slightly lower than those observed in cells overexpressing WT BMRP. Expression studies performed in HEK 293T cells show that the two BMRP site-directed mutants are expressed in cells at levels similar to those of the WT BMRP protein (Fig. 4C). Reversible Ponceau S staining of the nitrocellulose membrane previous to immunoblotting, and immunoblotting with an anti-α-tubulin Ab (Fig. 4D), were utilized to confirm approximate equal loading of the protein extracts used for the WB. The cell death induced by overexpression of the BMRP(D16A) mutant was blocked by coexpression of the baculoviral caspase inhibitor p35 protein (Fig. 4E). Bcl-2 also counteracted the cell death induced by BMRP(D16A), although the protection afforded by Bcl-2 to cells induced to die by BMRP(D16A) overexpression seemed to be somewhat lower than that provided to cells overexpressing WT BMRP (Fig. 4F). Morphological studies performed in NIH/3T3 cells show that the cell death induced by overexpression of the site-directed mutants BMRP(13Ala17) and BMRP(D16A) exhibits the characteristic morphological features of apoptosis (shrinkage of the cell, condensation of the nuclear chromatin, blebbing of the plasma membrane, and fragmentation of the cell into apoptotic bodies), as observed for cells undergoing cell death by overexpression of WT BMRP (Fig. 5 and Fig. S1). Bad, a pro-apoptotic member of the Bcl-2 family of proteins [Yang et al., 1995; del Peso et al., 1997; Youle and Strasser, 2008], was used as control for comparison. Quantitation of cells positive for nuclear AcGFP1 (GFPnuc positive cells) 40 and 60 h post-transfection showed that WT BMRP overexpression causes a reduction in the number of fluorescent cells, as expected from its ability to induce apoptotic cell death (Fig. 6). Collectively, the results obtained in these functional analyses suggest that BMRP does not induce apoptosis by binding to and blocking the anti-apoptotic activity of Bcl-2, since the Ala substitution BMRP mutants BMRP(13Ala17) and BMRP(D16A), which have a significantly reduced capacity to bind to Bcl-2, are at least as potent in inducing apoptosis as WT BMRP. A more likely possibility is that Bcl-2 binding to BMRP results in a partial reduction of the pro-apoptotic activity of BMRP, hence its moderately reduced ability to block BMRP(D16A) effects.

DISCUSSION

Numerous ribosomal proteins have been reported to participate in processes other than protein biosynthesis. These alternative roles of ribosomal proteins include the regulation of cell growth, death, and transformation [Lindstrom, 2009; Warner and McIntosh, 2009]. Cytosolic ribosomal proteins that have been shown to be involved in apoptosis comprise RPL5, RPL11, RPL23, RPS7, L7, L13a, S3a, and S29 [Naora et al., 1998; Khanna et al., 2000; Lindstrom, 2009; Warner and McIntosh, 2009]. Three mitochondrial ribosomal proteins have also been reported to play a role in the regulation of cell death by apoptosis: DAP3, PDCD9, and more recently, BMRP [Kissil et al., 1999; Cavdar Kov et al., 2001; Mukamel and Kimchi, 2004; Chintharlapalli et al., 2005; Kim et al., 2007; Tang et al., 2009]. BMRP was identified as a Bcl-2 binding partner using a yeast Two-Hybrid approach, and shown to have pro-apoptotic activity [Chintharlapalli et al., 2005; Malladi et al., 2011]. Bcl-2 counteracts

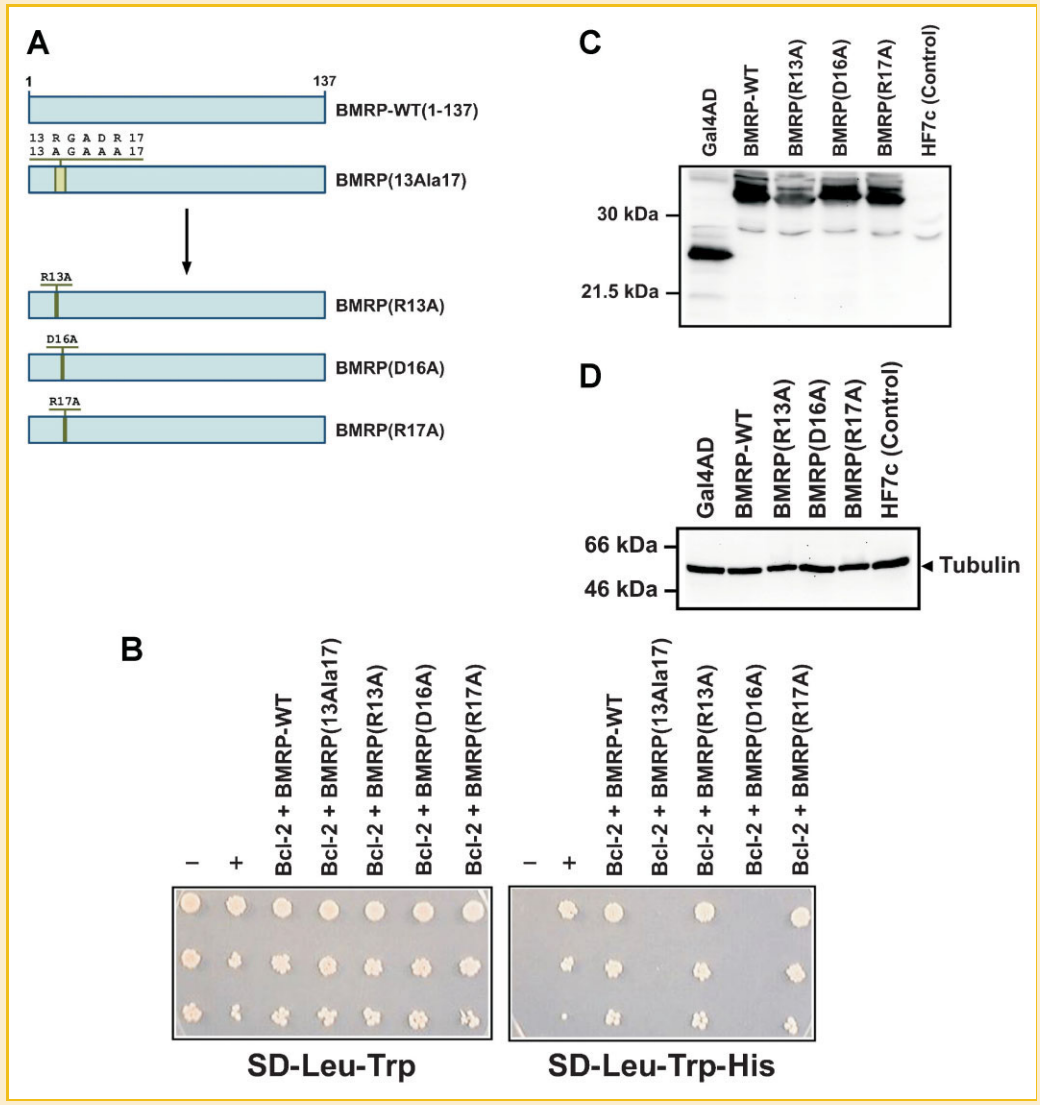

Fig. 3. Analysis of the binding of BMRP single Ala substitution mutants to Bcl-2 in yeast cells. A: Schematic diagram of the three BMRP point Ala substitution mutants generated (BMRP(R13A), BMRP(D16A), and BMRP(R17A)). WT BMRP and BMRP(13Ala17) are also shown for comparison. B: Yeast Two-Hybrid assay performed using WT BMRP, or the BMRP single Ala substitution mutants, fused to Gal4AD, and Bcl-2Δ13 fused to Gal4BD. The yeast strain HF7c was cotransformed with yeast Two-Hybrid vectors encoding WT BMRP or its single amino acid mutants, BMRP(R13A), BMRP(D16A), or BMRP(R17A), and Bcl-2Δ13. The cotransformants were selected, and then spotted on SD-Leu-Trp and SD-Leu-Trp-His plates as indicated in Figure 2B. (−), Negative control: HF7c cells cotransformed with the plasmid encoding the Gal4DBD/Bcl-2Δ13 fusion protein and the empty pGAD-GH plasmid. (+), The interaction between Bcl-2Δ13 and Bad proteins was utilized as a positive control in this assay. The results show that the BMRP(D16A) mutant is unable to interact with Bcl-2Δ13, suggesting that amino acid residue D16 of BMRP is required for the BMRP/Bcl-2 interaction in yeast cells. Results are representative of six independent experiments. C: Analysis of the expression of Gal4AD/BMRP point mutants in yeast cells. Protein extracts from HF7c cotransformed cells expressing Gal4AD (negative control) or Gal4AD/WTBMRP (positive control), or its point Ala substitution mutants, were prepared and resolved on a SDS-12% polyacrylamide gel. Immunoblotting utilizing rabbit anti-Gal4AD serum demonstrated the expression of all BMRP point mutant hybrid proteins. The predicted molecular mass of the proteins is as indicated in Figure 2C. Results are representative of three independent experiments. D: Equal loading of the lysates analyzed by WB in part (C) was confirmed by immunoblotting of these lysates with mouse anti-α-tubulin Ab. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

BMRP-induced cell death, and coexpression of p35, a baculoviral caspase inhibitor, also blocks cell death caused by BMRP overexpression. Additionally, BMRP has been reported to participate in apoptosis by inhibiting the ubiquitination of p53 and enhancing its translocation to the mitochondria, where p53 induces apoptosis via transcription-independent mechanisms [Yoo et al., 2005]. A tumor-supressor role for BMRP has been suggested based on its ability to inhibit the growth of cancer cells in tissue culture and of tumors in nude mice, as well as its low or undetectable levels of expression in various tumor tissues [Yoo et al., 2005].

Deletion mutational analyses indicated that the region(s) of BMRP required for its interaction with Bcl-2 reside in the N-terminal two-thirds of the BMRP protein [Malladi et al., 2011]. These studies also showed that the N-terminal two-thirds of BMRP are mainly responsible for its pro-apoptotic activity. We have continued our characterization of the BMRP/Bcl-2 interaction and the pro-

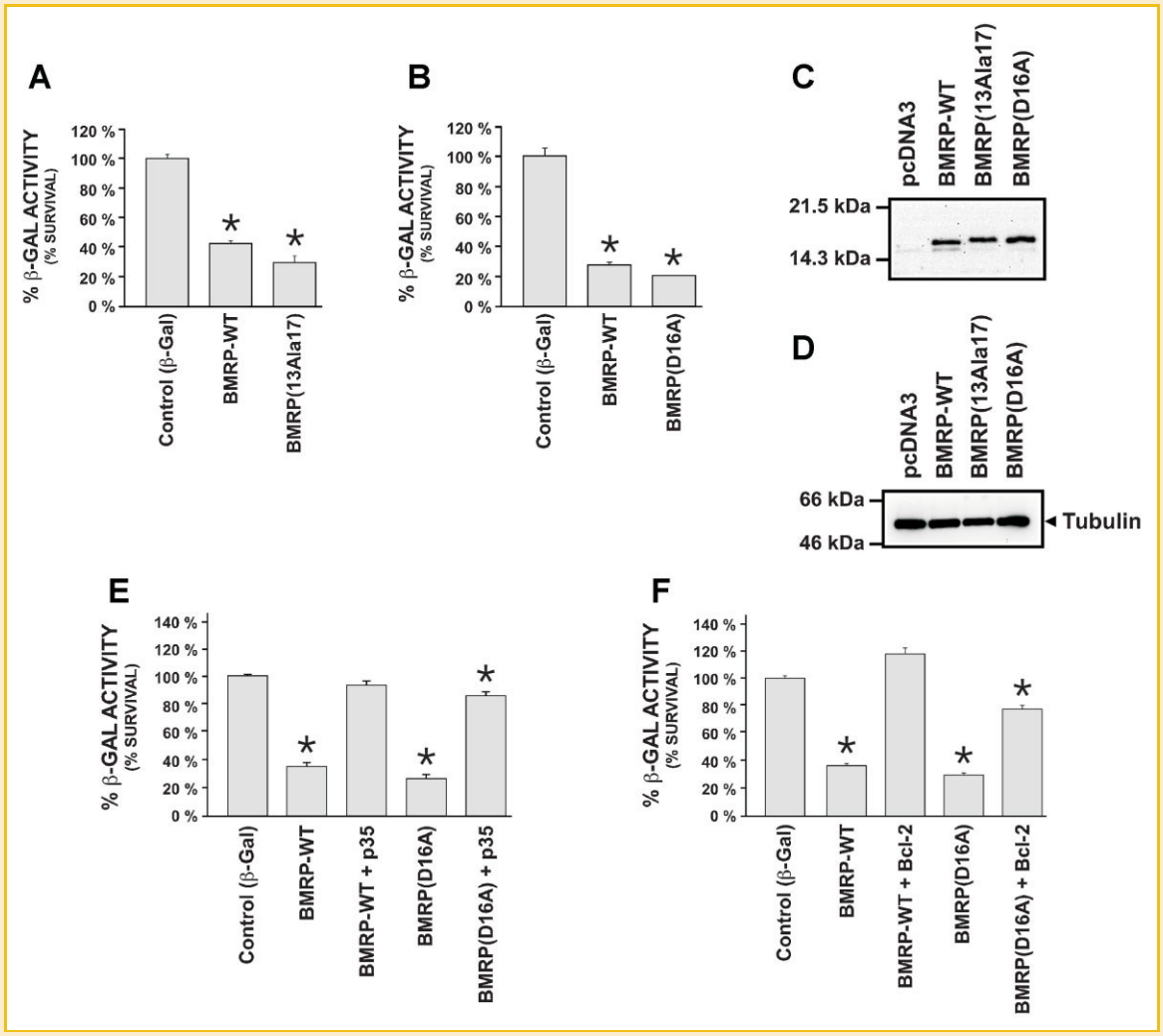

Fig. 4. Functional characterization of the BMRP(13Ala17) and BMRP(D16A) mutants. A: Overexpression of BMRP(13Ala17) induces cell death. NIH/3T3 cells were transiently transfected with eukaryotic expression vectors (400 ng) producing the indicated proteins along with a smaller amount of plasmid encoding β-galactosidase as a reporter (200 ng). Cell survival was quantified 48 h after transfection by determining β-galactosidase activity. A value of 100% has been assigned to the survival of the cells transfected with empty vector. The results shown are representative of two different experiments, each one performed in triplicate. B: Overexpression of BMRP(D16A) promotes cell death. NIH/3T3 cells were used for these experiments as described for part (A). The experiment presented is representative of three independent experiments, each performed in triplicate. C: WB analysis of BMRP site-directed mutants in HEK 293T cells. HEK 293T cells were transiently transfected with eukaryotic expression vectors driving the expression of the indicated proteins. The control was transfected with empty vector. Cell lysates were resolved on a SDS-12% polyacrylamide gel. The expression of the site-directed BMRP mutants BMRP(13Ala17) and BMRP(D16A) was detected in mammalian cells by immunoblotting (performed with a 1:1,000 dilution of rabbit polyclonal anti-BMRP serum). The predicted molecular mass of the WT BMRP, BMRP(13Ala17) and BMRP(D16A) proteins is 15.4 kDa. Results are representative of two independent experiments. D: Equal loading of the HEK 293T protein extracts in the WB shown in part (C) was confirmed by immunoblotting with mouse anti-α-tubulin Ab. E: p35 rescues cells from BMRP(D16A)-induced cell death. Cell viability reduction assays in NIH/3T3 cells showed that p35 blocks the pro-apoptotic activity of BMRP(D16A), suggesting that BMRP(D16A) kills cells by a mechanism similar to that of WT BMRP (involving caspase activity). The results shown are representative of three independent experiments, each performed in triplicate. F: Bcl-2 counteracts BMRP(D16A)-induced cell death. Coexpression of Bcl-2 protected cells from death caused by BMRP(D16A) overexpression; however, this protection was not as complete as that observed for WT BMRP-induced cell death. The experiment shown is representative of two independent experiments, each performed in triplicate. For graphs shown in panels (A), (B), (E), and (F), statistically significant decrease in cell viability versus the Control (β-gal) is indicated with an asterisk ($P < 0.01$).

apoptotic function of BMRP by performing site-directed mutagenesis analyses of the BMRP protein. Six short stretches of amino acids within the N-terminal two-thirds of BMRP were selected for Ala substitution mutagenesis, based on the sequence homology observed in the alignment of the hBMRP protein with the BMRP proteins from eight other species. Binding analyses of the six mutants generated revealed that the 13–17 amino acid region of BMRP is required for its interaction with Bcl-2. Individual amino acid substitutions within the 13–17 motif indicated that amino acid residue D16 of BMRP is essential for its binding to Bcl-2. The expression of all the Gal4AD hybrid proteins in yeast cells was confirmed by WB. The predicted molecular mass of Gal4AD is 21 kDa; however, this protein migrates slower in SDS–PAGE (Fig. 2C and Fig. 3C). The anomalous migration of some proteins in SDS–PAGE has been studied thoroughly, and is known to occur for proteins that have unusual

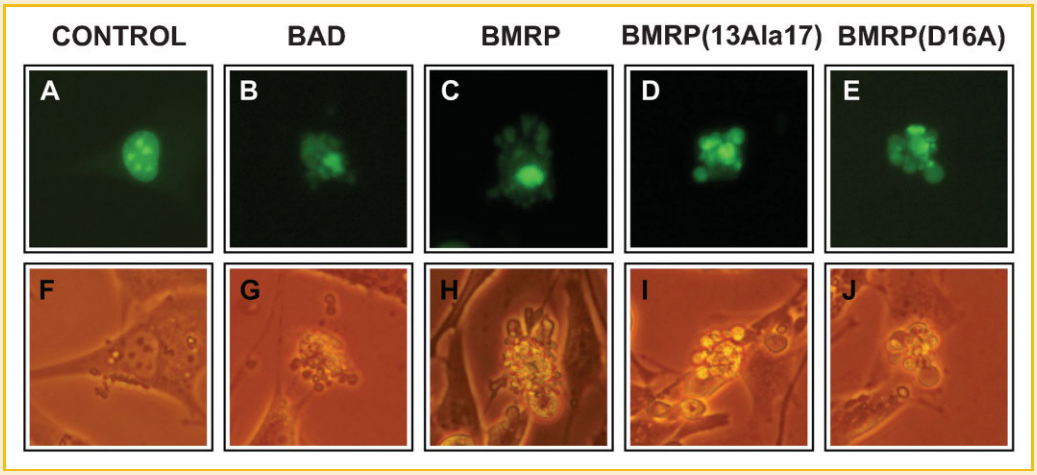

Fig. 5. BMRP(13Ala17)- and BMRP(D16A)-induced cell death exhibit the morphological hallmarks of apoptosis. NIH/3T3 cells undergoing cell death by BMRP(13Ala17) or BMRP(D16A) overexpression are shrunken, exhibit nuclear chromatin condensation and plasma membrane blebbing, and are fragmenting into apoptotic bodies, some of which are being released from the remainder of the cell (panels D,I and E,J, respectively). These morphological features are similar to those show by NIH/3T3 cells undergoing death by WT BMRP (panels C,H), or Bad (panels B,G) overexpression. Bad is a well-characterized pro-apoptotic protein that belongs to the Bcl-2 family of proteins. Control cells (transfected with empty plasmid) exhibit normal morphology (panels A and F). Photomicrographs (F–J) are the phase-contrast images corresponding to the fluorescent photomicrographs shown in panels (A–E). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

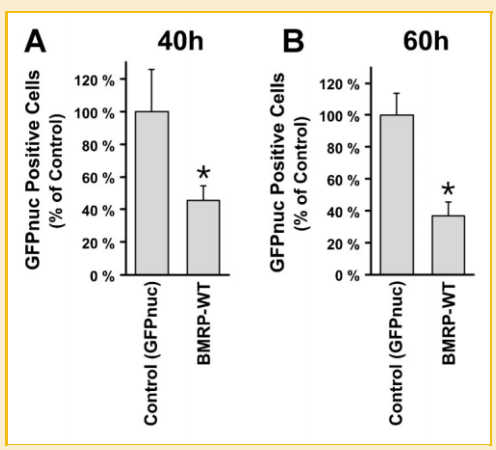

Fig. 6. Overexpression of WT BMRP results in cell death of cells coexpressing nuclear AcGFP1. A: Reduction in the number of GFPnuc positive cells caused by BMRP coexpression 40 h post-transfection. The number of cells expressing high levels of nuclear AcGFP1 in NIH/3T3 cells cotransfected with plasmids encoding WT BMRP and nuclear AcGFP1 was determined by fluorescence microscopy coupled with image analysis. The results obtained were normalized to the control (transfected with the nuclear AcGFP1 expressing plasmid and the pcDNA3 empty plasmid). The number of GFPnuc positive cells for the cotransfection with the plasmid encoding WT BMRP was 45.8% ± 8.8% relative to the control transfection. Statistically significant decrease of GFPnuc positive cells in BMRP-WT samples versus the Control (GFPnuc) is indicated with an asterisk ($P < 0.05$). B: Reduction in the number of GFPnuc positive cells caused by BMRP coexpression 60 h post-transfection. A statistically significant ($P < 0.05$) decrease of cells positive for nuclear AcGFP1 was observed in the BMRP-WT sample, with a percentage of 37.1% ± 8.5% versus the control. The decrease in the ratio of GFPnuc positive cells observed from 40 to 60 h suggests that a number of these NIH/3T3 cells have undergone apoptotic death. The results shown in panels (A) and (B) are representative of two different experiments, each performed in triplicate.

charge (with acidic or basic pIs), proteins that contain carbohydrate moieties (such as glycoproteins), proteins rich in hydrophobic regions (such as membrane proteins), and proteins that contain unreduced disulfide bonds. Gal4AD is an acidic protein with a calculated pI of 4.49. Several acidic proteins have been reported to show slow migration in SDS–PAGE, including GAP43, gRICH68/70, caldesmon, etc., probably due to the low binding of the negatively charged SDS detergent to these proteins because of repulsion effects [Banker and Cotman, 1972; Ballestero et al., 1997; Garcia-Ortega et al., 2005].

One possible mechanism by which BMRP could induce apoptosis is by interacting with Bcl-2 and blocking its anti-apoptotic activity. Other pro-apoptotic proteins promote cell death by binding to and blocking the anti-apoptotic function of Bcl-2 such as Bad [Yang et al., 1995; del Peso et al., 1997; Youle and Strasser, 2008]. To test this hypothesis, the ability of the BMRP site-directed mutants BMRP(13Ala17) and BMRP(D16A) to promote cell death was analyzed in NIH/3T3 cells. The results obtained indicate that these mutants induce apoptosis, and that they kill cells slightly more potently than WT BMRP. Thus, BMRP does not seem to promote apoptosis by binding and blocking the anti-apoptotic activity of Bcl-2. The available data is more consistent with a model in which binding of Bcl-2 to BMRP results in a partial reduction of the pro-apoptotic activity of BMRP. This model would explain that the BMRP(13Ala17) and BMRP(D16A) mutants are slightly more potent than WT BMRP in promoting apoptosis, and that Bcl-2 shows a moderately reduced ability to counteract BMRP(D16A) versus WT BMRP effects. With the results observed, there is still the possibility that the interaction detected in the yeast Two-Hybrid system does not play an important role in the function of BMRP in apoptosis; however, it should be emphasized that in complex signaling systems blocking only one of the branches may not result in an observable effect, particularly when using overexpression model systems. For

example, growth factor receptor tyrosine kinases have been shown to regulate and interact with various proteins involved in several intracellular signaling pathways related to cell growth and differentiation, which made it difficult to pinpoint the role of individual signaling proteins in the effects of the growth factors [Lemmon and Schlessinger, 2010]. Bcl-2 is involved in a highly complex signaling system. Therefore, it is not surprising that Bcl-2 is able to prevent a very significant portion of the cell death caused by overexpression of BMRP(D16A), since it is well known that Bcl-2 can employ several mechanisms to exert its anti-apoptotic function. One of these mechanisms involves the binding and neutralization of the pro-apoptotic activities of BH3-only proteins, such as Bad, Bim, Puma, and tBid [Yang et al., 1995; del Peso et al., 1997; Youle and Strasser, 2008]. The BH3-only proteins constitute one sub-group of the Bcl-2 family that share homology only at the level of the BH3 domain, and are the most powerful apoptosis inducers of the family [Adams and Cory, 2001; Youle and Strasser, 2008]. High levels of Bcl-2 can prevent the activation of Bax and Bak, blocking the mitochondrial outer membrane permeabilization (MOMP), the activation of caspases, and, ultimately, the dismantling of the cell by apoptosis [Kluck et al., 1997; Adams and Cory, 2001; Danial and Korsmeyer, 2004; Lucken-Ardjomande and Martinou, 2005; Youle and Strasser, 2008]. Bcl-2 has also been reported to interact with several other proteins, such as the non-mammalian pro-apoptotic protein Ced-4, the transcription factor NF-κB, the protein kinase Raf-1, and the inositol 1,4,5-triphosphate receptor (IP3R), mediating different processes leading to cell survival [Huang et al., 1998; Rong et al., 2009]. Therefore, overexpressed levels of Bcl-2, by activating all these different survival pathways, do overcome most of the death promoting effects caused by BMRP(D16A) over-expression, despite the fact that Bcl-2 cannot bind and sequester this BMRP site-directed mutant. An in vitro assay with pure proteins could discern whether the binding of Bcl-2 to BMRP results in biochemical effects on the function of BMRP. These assays were successful in detecting very specific effects for the cytochrome c interaction with Apaf-1 [Zou et al., 1997]; however, these assays cannot be designed yet for BMRP, as its mechanisms of triggering apoptosis are still not well understood. Since the amino acid residue D16 of BMRP, which has been found to be essential to the BMRP/Bcl-2 interaction, does not seem to be necessary for the pro-apoptotic activity of BMRP, it is important to conduct additional site-directed mutagenesis studies to identify the motif(s) of BMRP that are relevant to its cell death inducing activity.

Another interesting question regarding the physiological relevance of the interaction of BMRP with Bcl-2 relates to where does the interaction take place in the cell. The current view is that Bcl-2 resides mainly on the outer mitochondrial membrane (but also on the membrane of the ER and the outer nuclear membrane). BMRP is a mitochondrial matrix protein, which leads to the question of how can it interact with Bcl-2 in eukaryotic cells. Several studies have shown partial localization of Bcl-2 to the inner mitochondrial membrane ([Gotow et al., 2000], and references cited within), which could allow interaction with a matrix protein. However, based on the fact that the interaction seems to be mediated by residues near the amino terminus of BMRP, a likely possibility is that the interaction with Bcl-2 occurs in the cytosol with the pre-BMRP protein (prior to import into the mitochondrion), rather than with the mature protein. Based on our unpublished observations that suggest that the mature protein does not interact with Bcl-2, and that N-terminal BMRP fusion proteins, which are probably not imported into the mitochondria, are still capable of inducing cell death, it could be speculated that accumulation of pre-BMRP in the cytosol may serve as an apoptosis trigger. In this scenario, Bcl-2 could interact with pre-BMRP and block its action at low levels, but would be overwhelmed in overexpressing cells. Further studies with mutant versions of the protein that prevent mitochondrial import, yet are still capable of interaction with Bcl-2, would be useful to test this hypothesis. As indicated above, a combination of overexpression and biochemical studies may be required to explore the mechanism and effects of the interaction of BMRP with Bcl-2. These studies should enhance our understanding of the mechanisms by which BMRP promotes apoptosis and of the signaling pathways involved in apoptosis in mammalian cells, which would provide novel targets for the development of anti-cancer therapies.

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