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BMRP Is a Bcl-2 Binding Protein That Induces Apoptosis

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Abstract Members of the Bcl-2 family of proteins play important roles in the regulation of cell death by apoptosis. The yeast Two-Hybrid system was utilized to identify a protein that interacts with the anti-apoptotic protein Bcl-2, designated BMRP. This protein corresponds to a previously known mitochondrial ribosomal protein (MRPL41). Binding experiments confirmed the interaction of BMRP to Bcl-2 in mammalian cells. Subcellular fractionation by differential centrifugation studies showed that both Bcl-2 and BMRP are localized to the same fractions (fractions that are rich in mitochondria). Northern blot analysis revealed a major *bmrp* mRNA band of approximately 0.8 kb in several human tissues. Additionally, a larger 2.2 kb mRNA species was also observed in some tissues. Western blot analysis showed that endogenous BMRP runs as a band of 16–17 kDa in SDS–PAGE. Overexpression of BMRP induced cell death in primary embryonic fibroblasts and NIH/3T3 cells. Transfection of BMRP showed similar effects to those observed by overexpression of the pro-apoptotic proteins Bax or Bad. BMRP-stimulated cell death was counteracted by co-expression of Bcl-2. The baculoviral caspase inhibitor p35 also protected cells from BMRP-induced cell death. These findings suggest that BMRP is a mitochondrial ribosomal protein involved in the regulation of cell death by apoptosis, probably affecting pathways mediated by Bcl-2 and caspases. J. Cell. Biochem. 94: 611–626, 2005.

Key words: Bcl-2; apoptosis; BMRP; mitochondria; ribosome; yeast Two-Hybrid screen

Apoptosis is a physiological type of cell death that is required for the development and normal function of metazoans. Deregulation of the tightly controlled apoptotic process has been shown to contribute to diseases such as cancer, neurodegenerative disorders, and diseases of the immune system [Vaux et al., 1988; Mattson, 2000; Starcevic et al., 2001; Green and Evan, 2002; Rathmell and Thompson, 2002; Macdonald et al., 2003]. Numerous proteins that participate in apoptosis have been identified. Among them, the members of the Bcl-2 family of proteins play important roles in the regulation of this program of cell suicide [Strasser et al., 2000; Adams and Cory, 2001; Borner, 2003]. The founder of this family, Bcl-2, was identified at the breakpoint of the t(14;18)(q32;q21) chromosomal translocation, which is the most common translocation associated with human lymphoid malignancies [Cleary et al., 1986]. Bcl-2 has been extensively studied, and has been shown to protect cells from apoptosis elicited by a diverse array of stimuli [Strasser et al., 1991; Garcia et al., 1992; Reed, 1994]. The inability of Bcl-2 to protect cells from apoptosis induced by some stimuli led to the identification

Abbreviations used: AD, activation domain; Bcl-2, B-cell leukemia/lymphoma 2; BD, DNA binding domain; BH domain, Bcl-2 homology domain; BMRP, Bcl-2 interacting mitochondrial ribosomal protein L41; CyF, cytosolic fraction; ER, endoplasmic reticulum; EST, expressed sequence tag; HEK 293T, human embryonic kidney 293T; HEL 299, human embryonic lung 299; HMF, heavy membrane fraction; IRES, internal ribosome entry site element; LMF, light membrane fraction; MEF, mouse embryo fibroblast; MISP, mitochondrial import signal peptide; MYS Cl2 BCF1, mouse embryo yolk sac Cl2 BCF1; P1F, pellet 1 fraction; PTP, permeability transition pore; OD, optical density; ORF, open reading frame; SD, synthetic defined; UTR, untranslated region; VDAC, voltage-dependent anion channel.

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of Bcl-x [Sentman et al., 1991; Boise et al., 1993; Cuende et al., 1993], another very powerful inhibitor of apoptosis [Boise et al., 1993; Gonzalez-Garcia et al., 1995]. Several other proteins with structural similarity to Bcl-2 have been identified since. Members of the Bcl-2 family of proteins share at least one of four BH (Bcl-2 Homology) domains, BH1 through BH4, and several members also contain a transmembrane domain that is located at the carboxyl terminus.

The Bcl-2 family comprises both anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, A1, ...) and pro-apoptotic proteins. The pro-apoptotic subfamily is further subdivided into two groups, the Bax-like (Bax, Bak, Bcl-xs, ...) and the BH3-only (Bid, Bad, Hrk, ...) group. The survival factors contain at least three or all four BH regions (except some viral members of this subfamily, which contain two BH domains). The proteins of the Bax-like group, more distantly related to Bcl-2, contain two or three BH regions. The BH3-only proteins are the most powerful apoptosis inducers, and share homology only at the level of the BH3 domain. The transmembrane domain that is present in many of the Bcl-2 family members is believed to mediate attachment to different organellar membranes including the outer mitochondrial membrane, the nuclear envelope, and endoplasmic reticulum (ER) membrane [Gonzalez-Garcia et al., 1994; Strasser et al., 2000; Adams and Cory, 2001; Borner, 2003]. This subcellular localization seems to be fixed for some members of the family (anti-apoptotic), while it appears to change for others (pro-apoptotic) upon the receipt of an apoptotic signal. For example, Bax has been shown to be mostly cytosolic in viable cells and then translocate to the mitochondrial membrane once the cell is signaled to undergo apoptosis [Adams and Cory, 2001]. Anti-apoptotic and pro-apoptotic members of the Bcl-2 family of proteins have the ability to heterodimerize. These dimerizations appear to be mediated by the BH1, BH2, and BH3 domains. These three domains were shown to form an elongated hydrophobic cleft in Bcl-xL, where a second BH3 amphipathic α helix can bind [Borner, 2003]. The activities of pro-apoptotic family members have been shown to be regulated by post-translational modifications. For example, the pro-apoptotic function of Bad is regulated by phosphorylation carried out by the protein kinase Akt [del Peso et al., 1997], and Bid is activated by Caspase-8-mediated proteolysis [Strasser et al., 2000].

The biochemical mode of action of mammalian anti-apoptotic Bcl-2 family members is still under intense investigation. It is likely that Bcl-2 and other mammalian homologs promote survival by binding and blocking the action of Ced-4 mammalian homologs as it has been shown in *Caenorhabditis elegans*. Ced-9, the nematode's Bcl-2 homolog, prevents programmed cell death in this organism by binding to and sequestering Ced-4 to mitochondria, thus preventing Ced-4 from activating pro-Ced-3 [Wu et al., 1997; Chen et al., 2000]. A similar scenario is likely to occur in mammals since Bcl-2 can functionally substitute for Ced-9 in *C. elegans* [Vaux et al., 1992; Hengartner and Horvitz, 1994]. Apaf-1, a Ced-4 homolog, was initially believed to bind to Bcl-xL on the external mitochondrial membrane in the absence of an apoptotic stimulus, holding pro-caspase 9 in an inactive state. However, this binding is still not fully confirmed. Other mammalian Ced-4 homologs are likely to exist since the phenotype of Apaf-1 knock-out mice is largely restricted to neurons [Honarpour et al., 2000], and Bcl-2 can still protect Apaf-1 deficient embryonic stem cells from death stimuli [Haraguchi et al., 2000]. Additionally, Bcl-2 and Bcl-xL have been shown to inhibit the release of cytochrome c from the mitochondrial intermembrane space to the cytosol, which occurs following a variety of apoptotic stimuli [Kluck et al., 1997; Shimizu et al., 1999]. Thus, mammalian pro-survival factors probably provide stability to mitochondria, thereby precluding the exit of apoptogenic factors to the cytosol (cytochrome c, AIF, Smac/Diablo, some procaspases, etc.). This function may be accomplished by interaction of anti-apoptotic factors with the voltage-dependent anion channel (VDAC) component of the permeability transition pore (PTP), or by some other still unknown mechanism [Shimizu et al., 1999].

To fully understand the mode of action of a protein, knowledge of all the proteins that interact with it is necessary. With this purpose, a search of proteins that bind to Bcl-2 was performed utilizing a yeast Two-Hybrid assay. A novel protein that interacts with Bcl-2 was identified in this screening. The protein was originally designated as BBP (Bcl-2 binding protein) based on its ability to interact with Bcl-2. Upon further alignment studies with cDNAs

and expressed sequence tag (EST) sequences, this protein was found to correspond to human mitochondrial ribosomal protein L41 (MRPL41; also designated as MRPL27 in one report) [Goldschmidt-Reisin et al., 1998; Kenmochi et al., 2001]. The name BBP/MRPL41, or BMRP (Bcl-2 interacting mitochondrial ribosomal protein L41), will be utilized throughout this article to indicate that this protein has the ability to interact with Bcl-2. In the present report, the Bcl-2/BMRP binding observed in yeast has been confirmed in mammalian cells by co-immunoprecipitation studies. Subcellular fractionation experiments show that BMRP and Bcl-2 localize to the same fractions, mainly the mitochondrial fractions. Expression of BMRP induces cell death in several mammalian cell lines (including primary embryonic fibroblasts of mice and human origin, and NIH/3T3 cells), which is counteracted by Bcl-2. Baculoviral p35, an inhibitor of caspases, also represses BMRP-induced cell death. Taken together, these results suggest that BMRP is a novel protein involved in the regulation of apoptosis, which probably carries out its function by interacting with anti-apoptotic Bcl-2.

MATERIALS AND METHODS

Identification of BBP Utilizing the Yeast Two-Hybrid System

The yeast strain HF7c was transformed with the bait vector pGBT-8-hbcl-2, and then used to screen a human HeLa cDNA library fused to the prey vector pGAD-GH (Clontech, Palo Alto, CA). Cotransformed colonies containing potential interacting hybrid proteins were selected by plating on synthetic defined (SD) medium lacking leucine, tryptophan, and histidine. The His^+ transformants obtained were tested for expression of the second reporter gene (*lacZ*) using a filter assay for β-galactosidase activity. Approximately 3×10^6 clones were screened. Plasmids from positive yeast clones were isolated, and the AD/library plasmid selected by using its *LEU2* marker gene to complement an *E. coli leuB* mutation (strain HB101). Inserts from isolated plasmids were subjected to restriction analysis and nucleotide sequencing using the dideoxynucleotide chain-termination method. Isolated AD/library plasmids were retransformed into HF7c yeast cells in combination with empty pGBT8, pGBT-8-hbcl-2 and other negative control bait plasmids to discard false positives.

Plasmids

The yeast Two-Hybrid plasmid pGBT8-hbcl-2 has been previously described [Fernandez-Sarabia and Bischoff, 1993]. The plasmid pGAD10-hbad utilized as a positive control in yeast Two-Hybrid assays was isolated in previous screenings, and encodes an N-terminal truncated human Bad protein starting at amino acid 143 (143–204) fused to Gal4AD. pGAD-GH-hbmrp* is the plasmid isolated from the human HeLa cDNA library and codes for the Gal4AD/BMRP* protein. This hybrid protein contains 35 extra amino acids between the Gal4AD sequence and the full-length BMRP protein, which are encoded, mostly, by the 5′ untranslated region (UTR) of the *bmrp* cDNA. pGAD-GH-hbmrp was constructed by cloning the *bmrp* open reading frame (ORF) into the vector pGAD-GH. Therefore, the corresponding fusion protein encoded does not contain the 35 extra amino acids previously mentioned. PCR fragments containing the ORFs for hBMRP-C-Flag and hBMRP were subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA) generating the plasmids pcDNA3-C-Flag-hbmrp and pcDNA3-hbmrp, which encode a C-terminal Flag-tagged and wild type BMRP, respectively. The plasmid pSFFVNeo-hbcl-2 has been previously described [Boise et al., 1993]. Plasmids pcDNA3-HA-mbax, pcDNA3-AU1-mbad, and pcDNA3-p35 code for murine Bax (N-terminal HA-tagged), murine Bad (N-terminal AU1-tagged), and baculoviral p35 respectively (kindly provided by Dr. Luis del Peso and Dr. Gabriel Núñez). The reporter plasmid pcDNA3-β-galactosidase used in functional studies has been described elsewhere [del Peso et al., 1998].

Yeast Drop Assay

A single yeast colony obtained in the cotransformation procedure was resuspended in 500 μl of sterile H_2O, and the optical density (OD) of each cell suspension was determined at a wavelength of 600 nm. A cell suspension of each of the cotransformants was prepared at an OD_{600} value of 0.01 (500 μl final volume, diluted in H_2O). This initial cell suspension (OD_{600} of 0.01) was serially diluted twice (1/5 dilution at each step). Five microliters of the initial suspension (0.01 OD_{600}) and each of the two dilutions were spotted on SD-leu-trp and SD-leu-trp-his plates. The culture plates were incubated at

30°C for 2–3 days. At least 20 different colonies expressing each pair of fusion proteins were tested.

Alignment of Two cDNA and Several EST Clones to Obtain an Extended BMRP cDNA

The two human cDNA clones from the GenBank corresponding to MRPL41, BC040035 (GI: 25123179) and NM_032477 (GI: 21265092), were aligned with 21 human MRPL41 EST clones to generate an extended MRPL41/BMRP cDNA (Fig. 1B). The EST sequences selected extend the human MRPL41/BMRP cDNA by 26 bp at the 5′ UTR. The GenBank accession numbers corresponding to the EST sequences used are the following: BF224160, BF110922, BE674326, BF223518, AI435280, BE326258, BE549963, BE550220, BE550774, BE672517, BM919526, BQ653726, BQ644139, BQ652718, BE908283, BQ652524, BI761478, BQ685348, BI822692, BG491207, and BI915244. GeneJockeyII software (Biosoft, Cambridge, UK) was utilized to perform the alignment.

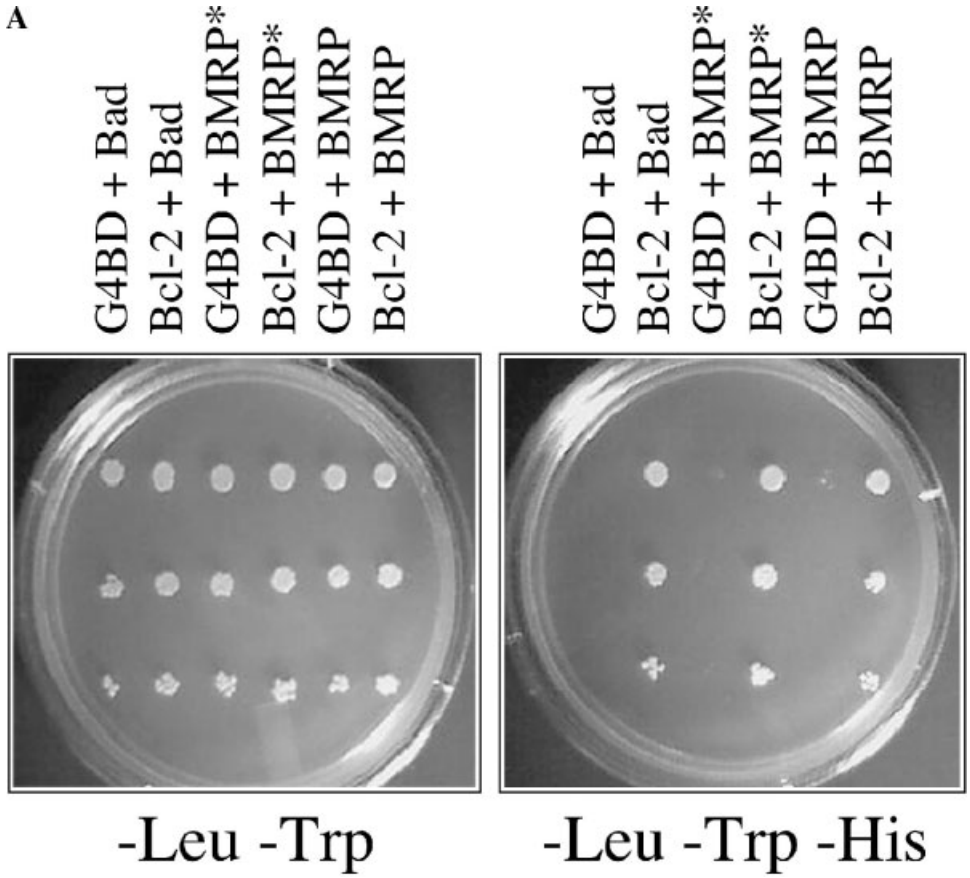
Expression of Recombinant BMRP Protein in Bacteria and Production of an Anti-BMRP Polyclonal Antibody

The coding region of the human *bmrp* gene, flanked by appropriate restriction sites, was amplified by PCR and subcloned into the prokaryotic expression vector pET-30a(+) Novagen, Madison, WI). The amplified PCR fragment was confirmed by sequencing using the dideoxynucleotide chain-termination method. This vector attaches a hexahistidine tag to the amino terminus of the translated protein, and allows isopropyl-1-thio-β-galactopyranoside-regulated expression of the recombinant hexahistidine-tagged BMRP protein in bacteria. The resulting plasmid, pET-30a(+)-hbmrp, was transformed into *Escherichia coli* (Bl21-CodonPlus™ [DE3] strain, Stratagene, La Jolla, CA). Lysis of bacteria expressing the recombinant BMRP protein was performed in ST-PIC buffer (200 mM NaCl, 20 mM Tris HCl pH 7; PIC is a protease inhibitor cocktail: 200 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) in the presence of 1% sarkosyl. Recombinant BMRP protein was purified from bacteria using a nickel-nitrilotriacetic column (Quiagen, Valencia, CA) as previously described [Ballestero et al., 1997]. Purified recombinant BMRP protein was injected into rabbits to obtain an anti-BMRP polyclonal antibody. Rabbit immunization was performed following standard protocols [Harlow and Lane, 1988]. Briefly, rabbits were injected subcutaneously with 275 μg each of purified BMRP protein, and then boosted at 2- (one time) and 4-week intervals (three times) with 137.5 μg of the purified protein for a total of four times. Test bleeds were collected at appropriate times to monitor the rabbits' immune response by performing ELISA assays and Western blots utilizing the purified recombinant BMRP protein.

Transient Transfection Into HEK 293T Cells, Co-Immunoprecipitation Studies and Western Blot Analysis

Human embryonic kidney 293T (HEK 293T) cells were transiently transfected (approximately 1.2×10^6 cells/well; multiwell-6 tissue culture plate) with eukaryotic expression vectors (1 μg of pSFFVNeo-hbcl-2, and 5 μg of pcDNA3-C-Flag-hbmrp alone or in combination) producing the corresponding proteins (see figure legends). Transfection was carried out by lipofection using Lipofectamine Plus reagent™ (Invitrogen) according to the manufacturer's recommendations. A negative control was trans-

Fig. 1. BMRP interacts with Bcl-2 in the yeast Two-Hybrid system. **A**: HF7c yeast cotransformant colonies were selected by plating on SD medium lacking leucine and tryptophan. A single yeast colony from each of the cotransformants was used 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. The plasmids pGBT8, pGBT8-hbcl-2, pGAD10-bad, pGAD-GH-hbmrp* (original plasmid obtained in the Two-Hybrid screening), and pGAD-GH-hbmrp were utilized to drive the expression of the proteins Gal4BD (G4BD), Gal4BD/Bcl-2 (Bcl-2), Gal4AD/Bad (Bad, amino acids 143–204), Gal4AD/BMRP* (BMRP*), and Gal4AD/BMRP (BMRP), respectively. The data presented is representative of more than 20 different colonies tested for each cotransformant. **B**: The sequence represents the consensus for the alignment of two human cDNAs and 21 ESTs corresponding to MRPL41/BMRP (see details in ''Materials and Methods'' section). The sequence contains the 3′-untranslated region, including the poly-A tail, partial 5′-untranslated region, and the full ORF for the 137-amino acid-long predicted BMRP protein, as represented below the DNA sequence with the one-letter amino acid code. The putative polyadenylation signal is underlined. The peptide sequence corresponding to the postulated MISP is also underlined. The sequence comprised by the two thick arrows was present in the original Two-Hybrid clone fused in frame with the Gal4AD sequence. The sequence delimited by the two thin arrows corresponds to that of MRPL41 cDNA clone BC040035, the longer of the two MRPL41 clones containing the complete ORF that are available in the GenBank.



B

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                                                                    60
TGCGGCCGCGGCCAATCGGAGCCGCTCTTGCTGCGACGCAGCGGTCGGAAGCGGAGCAAG
                                                                   120
GTCGAGGCCGGGTTGGCGCCGGAGCCGGGGCCGCTTGGAGCTCGTGTGGGGTCTCCGGTC
                                                                   180
CAGGGCGCGGCATGGGCGTCCTGGCCGCAGCGGCGCGCTGCCTGGTCCGGGGTGCGGACC
  1                M  G  V  L  A  A  A  A  R  C  L  V  R  G  A  D  R
                                                                   240
GAATGAGCAAGTGGACGAGCAAGCGGGGCCCGCGCAGCTTCAGGGGCCGCAAGGGCCGGG
 18    M  S  K  W  T  S  K  R  G  P  R  S  F  R  G  R  K  G  R  G
                                                                   300
GCGCCAAGGGCATCGGCTTCCTCACCTCGGGCTGGAGGTTCGTGCAGATCAAGGAGATGG
 38    A  K  G  I  G  F  L  T  S  G  W  R  F  V  Q  I  K  E  M  V
                                                                   360
TCCCGGAGTTCGTCGTCCCGGATCTGACCGGCTTCAAGCTCAAGCCCTACGTGAGCTACC
 58    P  E  F  V  V  P  D  L  T  G  F  K  L  K  P  Y  V  S  Y  L
                                                                   420
TCGCCCCTGAGAGCGAGGAGACGCCCCTGACGGCCGCGCAGCTCTTCAGCGAAGCCGTGG
 78    A  P  E  S  E  E  T  P  L  T  A  A  Q  L  F  S  E  A  V  A
                                                                   480
CGCCTGCCATCGAAAAGGACTTCAAGGACGGTACCTTCGACCCTGACAACCTGGAAAAGT
 98    P  A  I  E  K  D  F  K  D  G  T  F  D  P  D  N  L  E  K  Y
                                                                   540
ACGGCTTCGAGCCCACACAGGAGGGAAAGCTCTTCCAGCTCTACCCCAGGAACTTCCTGC
118    G  F  E  P  T  Q  E  G  K  L  F  Q  L  Y  P  R  N  F  L  R
                                                                   600
GCTAGCTGGGCGGGGGAGGGGCGGCCTGCCCTCATCTCATTTCTATTAAACGCCTTTGCC
       *
                                                                   647
AGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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cDNA Two-Hybrid

Fig. 1.

fected with empty vector. In all cases empty plasmid was added so that the total amount of DNA was 6 μg/well. Approximately 48 h after transfection, cells were rinsed once and then incubated with PBS containing 1% formaldehyde for 30 min at room temperature. Formaldehyde is a small reactive molecule, capable of rapidly permeating intact cells, that has been utilized as a useful means of probing intracellular close molecular interactions [Jackson, 1999; Hall and Struhl, 2002]. After formaldehyde treatment, the cells were rinsed once with PBS and lysates were prepared in NP40-PIC lysis buffer (NP40 lysis buffer is 140 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES pH 7.2, 1 mM EGTA, and 0.2% NP40). The lysates were cleared of debris by centrifugation. Then, the Bcl-2 protein was immunoprecipitated overnight using an anti-Bcl-2 monoclonal antibody (BD Pharmingen, San Diego, CA) at a concentration of 2 μg/ml, followed by incubation with protein A-agarose beads (Roche Molecular Biochemicals, Indianapolis, IN) at 4°C for 3 h (both steps were performed on a rocking platform). Equal amounts of the same lysates were subjected to immunoprecipitation with control IgG. The protein A-agarose beads were collected by low speed centrifugation, followed by three successive washes with NP40-PIC lysis buffer, and one wash with PBS. SDS loading buffer (1×) was added to the beads [Sambrook et al., 1989]. The beads containing the immunoclomplexes were incubated at 96°C for 20 min and then loaded into a 15% SDS–polyacrylamide gel for analysis by Western blot [Sambrook et al., 1989]. Western blotting was performed with a 1:200 dilution of rabbit polyclonal anti-BMRP serum as primary antibody. A 1:5,000 dilution of goat anti-rabbit-HRP (Invitrogen) was used as secondary antibody. The secondary antibody was detected by chemiluminiscence using ECL substrate (Amersham Pharmacia Biotech, Piscataway, NJ). The results were recorded with a Kodak 440 Imager Station (Eastman Kodak Company, Rochester, NY).

Subcellular Fractionation by Differential Centrifugation and Succinate Dehydrogenase Assay

Transient transfection of HEK 293T (approximately 1.2×10^6 cells/well; multiwell-6 tissue culture plate) was performed basically as stated above. Eukaryotic expression vectors (1 μg of pSFFVNeo-hbcl-2 and 5 μg of pcDNA3-hbmrp alone or in combination) encoding the corresponding proteins (see figure legends) were utilized in the transfections. A negative control was transfected with empty vector. In all cases, empty plasmid was added so that the total amount of DNA was 6 μg/plate. Approximately 48 h after transfection, cells were scraped from tissue culture dishes in 400 μl of buffer A-PIC (buffer A is 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 10 mM HEPES, pH 7.8) and Dounce-homogenized (25–30 strokes). The homogenate was first subjected to low speed centrifugation (1,000*g* for 10 min at 4°C) to sediment the largest particles. This first pellet (P1) should contain most of the nuclei, large plasma membrane fragments, and large mitochondria. Then the supernatant was centrifuged again at a higher speed (10,000*g* for 10 min, also at 4°C) to sediment the heavy membrane fraction (HMF, probably enriched in mitochondria, large lysosomes, and peroxisomes). The supernatant obtained was subjected to ultracentrifugation (100,000*g* for 1 h, 4°C) to separate the light membrane fraction (LMF, containing most of the microsomes and small vesicles) from the cytosolic fraction (CyF, enriched in ribosomes and soluble macromolecules). Equivalent amounts (by volume) of the subcellular fractionated samples were run on a 15% SDS–polyacrylamide gel and immunoblotted with anti-BMRP antibody (1:200 dilution of rabbit serum) as previously described. The nitrocellulose membranes were then stripped and reblotted with anti-Bcl-2 monoclonal antibody (1 μg/ml, BD Pharmingen).

The different subcellular fractions of transfected HEK 293T cells were assayed for succinate dehydrogenase activity as described [Rickwood and Harris, 1996], with some modifications. Briefly, equivalent amounts (by volume) of each fraction (P1F, HMF, LMF, and CyF) were employed in these assays. The reaction was carried out in 50 mM phosphate buffer at pH 7.4. The substrates succinate and INT (*p*-iodonitrotetrazolium violet) were utilized at a final concentration of 10 mM and 625 μg/ml, respectively. After addition of the INT substrate, the reaction mixtures were incubated for 3 h at 37°C. A value of 100% was assigned to the sum of succinate dehydrogenase activity obtained with the four different fractions (P1F, HMF, LMF, and CyF) proceeding from the same lysate, and percentage succinate dehydrogenase activity for each one of the fractions was calculated.

Northern Blot Analysis of *bmrp* mRNA

For riboprobe synthesis, a cDNA fragment containing the ORF for human BMRP was subcloned into the pGEM-3Z plasmid (Promega, Madison, WI). The resulting plasmid was digested with *Bam*HI and riboprobe synthesis was performed by transcription with SP6 RNA polymerase (Invitrogen) and (α-^{32}P)UTP (ICN) as the labeled precursor [Ballestero et al., 1995]. A multiple tissue Northern blot with poly A^+ RNA from several human tissues (Clontech) was hybridized with the *bmrp* riboprobe following standard protocols [Sambrook et al., 1989].

Non-radioactive Northern blotting was carried out using the DIG system (Roche Molecular Biochemicals). The plasmid pBluescriptKS-hbmrp was digested with *Bam*HI, and the digoxigenin labeled riboprobe was synthesized by transcription with T3 RNA polymerase and DIG-11-UTP as the labeled precursor. A second human multiple tissue Northern blot (Clontech) was hybridized with this non-radioactive probe. Hybridization was performed in DIG Easy Hyb buffer following the manufacturer's instructions. The blot was developed using an anti-digoxigenin antibody linked to alkaline phosphatase. CDP-star (Promega) was used as the chemiluminiscence substrate to detect phosphatase activity. Controls were carried out utilizing several amounts of sense *bmrp* RNA (300, 100, 30, and 10 pg) to determine the detection limit of the non-radioactive Northern blot. The procedure was able of detecting 10–30 pg of sense *bmrp* RNA target.

Protein Extract Preparation From Mouse Tissues

Mouse tissues were obtained from Pel-Freez Biologicals. Lysates from mouse tissues were prepared in STT-PIC (STT is ST containing 0.1% Triton-X-100) by Dounce-homogenization. Total protein concentration from each tissue lysate was determined using the Bradford reagent (Pierce, Rockford, IL). Two hundred micrograms of total protein from the extracts were separated by 15% SDS–PAGE, and immunoblotted with rabbit polyclonal anti-BMRP antibody as described above.

Cell Death Assays

MYS Cl 2 BCF1 (mouse embryo yolk sac; ATCC, CRL-9292), HEL 299 (human embryonic lung; ATCC, CCL-137), and NIH/3T3 (ATCC, CRL-1658) cells were cultured for transfection in multiwell-12 tissue culture plates ($3–4 \times 10^4$ cells/well). Approximately 24 h after seeding, cells were cotransfected with the indicated plasmids (see figure legends; 200 ng/well for MYS Cl2 BCF1 cells, and 300 ng/well for HEL 299 and NIH/3T3 cells) along with a smaller amount of pcDNA3-β-galactosidase as a reporter (50 ng/well for MYS Cl2 BCF1 cells, 150 ng/well for HEL 299 cells, and 100 ng/well for NIH/3T3 cells). Transfections were performed by lipofection (Lipofectamine Plus™ for MYS and HEL 299 cells, Lipofectamine™ 2000 for NIH/3T3 cells; both reagents from Invitrogen) following the manufacturer's recommendations. In all cases, empty plasmid was added so that the total amount of DNA was 700 ng/well for MYS Cl2 BCF1 and NIH/3T3 cells, and 750 ng/well for HEL 299 cells. Survival of transfected cells was measured by quantification of reporter gene activity (β-galactosidase activity) basically as described [del Peso et al., 1998]. Briefly, 48 h after transfection (60 h in the case of HEL 299) cell lysates were prepared in 200 μl of 1× reporter lysis buffer (Promega). For assay of β-galactosidase activity, 150 μl of each lysate were mixed with 150 μl of 2× ONPG buffer (7.4 M of *o*-nitrophenyl-β-D-galactopyranoside in 164 mM $Na_2HPO_4.7H_2O$, 36 mM NaH_2PO_4, 2 mM $MgCl_2$, and 100 mM β-mercaptoethanol). Sample tubes were incubated for 3 h (16 h for NIH/3T3 cells) at 37°C, and product formation was monitored by reading the optical density at a wavelength of 420 nm. A value of 100% survival was assigned to the β-galactosidase activity of cells transfected with empty vector, and the percent 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).

RESULTS

Yeast Two-Hybrid Screening

A HeLa cDNA yeast Two-Hybrid library was screened for Bcl-2 binding proteins using sequential transformation. Proteins that have already been shown to interact with Bcl-2 (such as Bad) were found to bind to the Gal4BD/Bcl-2 hybrid bait in these studies, validating the screening procedure. One clone that encoded a protein not previously reported to bind to Bcl-2

was also found in this screening (Fig. 1A). DNA restriction and sequencing analysis indicated that this clone contained a cDNA fragment of 623 bp ending in a polyA tail. Searches of nucleotide and protein databases revealed that this clone encompassed the coding sequence of a human mitochondrial protein, mitochondrial ribosomal protein L41 (MRPL41, also referred to as MRPL27 in one report) [Goldschmidt-Reisin et al., 1998; Kenmochi et al., 2001], in frame with the Gal4AD ORF (Fig. 1B). The MRPL41 protein is 137 amino acids long with a predicted molecular weight of approximately 15.5 kDa. The two MRPL41 cDNA sequences present in the GenBank have been extended with EST sequences to obtain a longer cDNA with an additional segment at the 5′ UTR (Fig. 1B). Since initially the protein encoded by the Two-Hybrid cDNA clone had been tentatively designated as BBP for Bcl-2 Binding Protein, the name BBP/MRPL41, or simply BMRP (Bcl-2 interacting Mitochondrial Ribosomal Protein L41), will be utilized to refer to this protein throughout this article. The original Two-Hybrid clone included 85 bp corresponding to the 5′ UTR present in the *bmrp* mRNA (Fig. 1B). Translation of this region along with sequences added in the subcloning procedure resulted in the presence of a 35 amino acid stretch located between the Gal4AD and the BMRP protein sequences in the hybrid protein (Gal4AD/BMRP* hybrid). A new prey vector containing just the BMRP coding sequence was constructed and utilized in Two-Hybrid studies (Gal4AD/BMRP hybrid). These experiments confirmed that BMRP binds to Bcl-2 in the yeast Two-Hybrid system (Fig. 1A). Additional studies performed in yeast indicated that BMRP does not interact with Bcl-xs, Bax, or Hrk in the Two-Hybrid system, while binding to Bcl-xL was weak (data not shown). BMRP does not contain any of the four domains that are present in members of the Bcl-2 family of proteins (BH1, BH2, BH3, and BH4), suggesting that BMRP does not belong to this family. BMRP is postulated to contain a mitochondrial import signal peptide (MISP) that is 13 amino acid residues long (Fig. 1B) [Goldschmidt-Reisin et al., 1998].

Association of Bcl-2 and BMRP in Mammalian Cells by Co-Immunoprecipitation Studies

Interaction studies were carried out utilizing a mammalian system, the cell line HEK 293T, in order to confirm the binding data obtained in the Two-Hybrid assay. HEK 293T cells were cotransfected transiently with plasmids expressing Bcl-2 and BMRP proteins. The expression of both proteins was confirmed by Western blotting (Fig. 2A). Cell extracts were immunoprecipitated using an anti-Bcl-2 antibody, and the presence of co-immunoprecipitated BMRP was assessed by Western blotting using an anti-BMRP polyclonal antibody. Figure 2B shows that the Bcl-2 immunoprecipitate contained BMRP. Appropriate controls carried out with a matching isotype control antibody, and with lysates containing only one of the overexpressed proteins, or neither of the two, demonstrate that this binding is specific. Overexpressed BMRP-C-Flag run as a protein doublet in the Western blotting procedure (Fig. 2A). The upper band shown in Figure 2A corresponds to the protein that co-immunoprecipitated with Bcl-2 in Figure 2B. Expression of untagged BMRP resulted in a single band in HEK 293T cells (Fig. 3). These observations suggest that the Flag tag confers some unstability to the protein in HEK 293T, and that the bottom band may be a degradation product unable to interact with Bcl-2.

Bcl-2 and BMRP Co-Localize Intracellularly in Mammalian Cells

BMRP has been isolated as a Bcl-2 binding protein and has previously been reported as a mitochondrial ribosomal protein. The anti-apoptotic protein Bcl-2 has been shown to locate mainly to the outer mitochondrial membrane, but also to the ER and nuclear membranes [Monaghan et al., 1992; Krajewski et al., 1993]. To investigate the intracellular localization of BMRP, and determine whether Bcl-2 and BMRP localize to similar subcellular fractions in mammalian cells, differential centrifugation studies were performed. HEK 293T cells overexpressing BMRP were lysed and subjected to a differential centrifugation protocol to separate the pellet 1 (P1), heavy membrane (HM), light membrane (LM), and cytosolic (Cy) fractions. The presence of BMRP in each one of these fractions was assessed by Western blot using the anti-BMRP antibody. The results obtained in these experiments show that the BMRP protein is most abundant in the P1 and HM fractions of cells (Fig. 3). Low levels of BMRP are present in the LM fraction, while this protein is undetectable in the Cy fraction. This

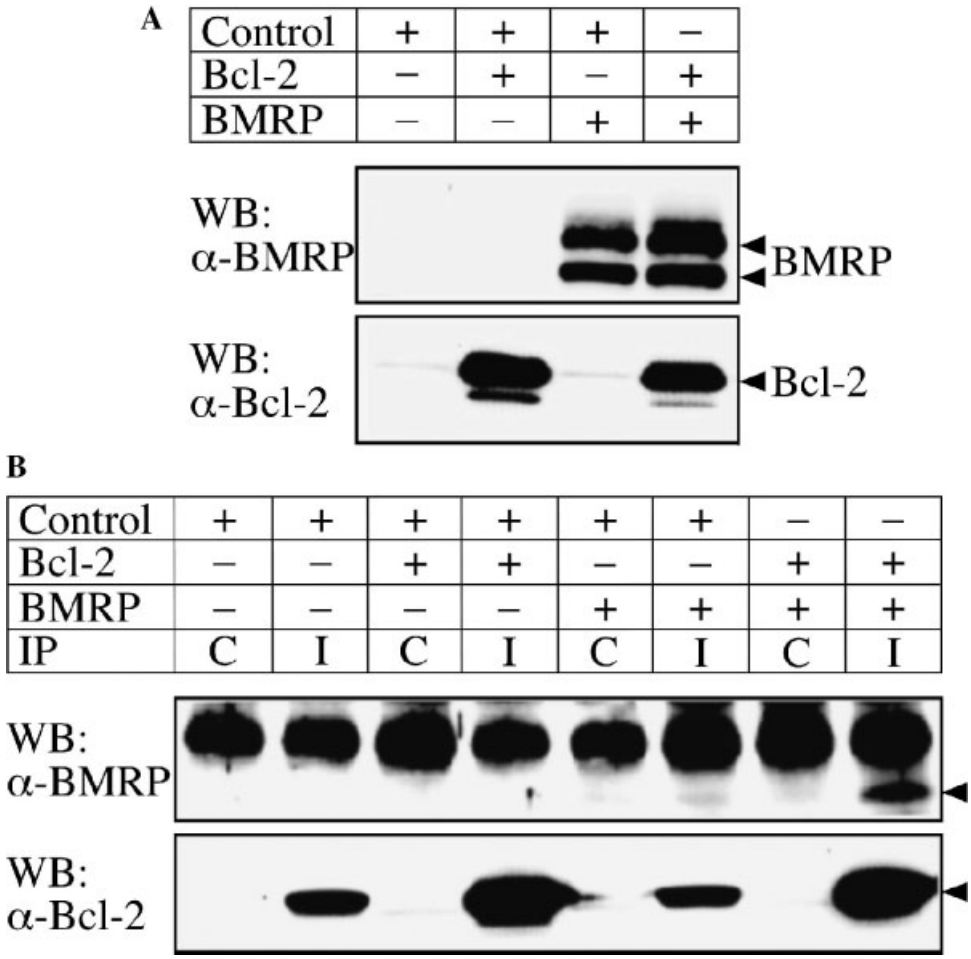

Fig. 2. Binding of BMRP to Bcl-2 in mammalian cells. **A**: HEK 293T cells were transiently transfected by lipofection with eukaryotic expression vectors producing the indicated proteins. The control was transfected with empty vector. Protein–protein interactions were fixed by cross-linking with 1% formaldehyde. Cell lysates were analyzed by Western blot with anti-BMRP antibody (WB: α-BMRP). The same membrane was then immunoblotted with anti-Bcl-2 antibody (WB: α-Bcl-2). Over-expressed BMRP-C-Flag protein in HEK 293T often appears as a doublet of approximately 16 and 18 kDa. **B**: Same cell lysates as in (A) were immunoprecipitated (IP) with anti-Bcl-2 mAb and then immunoblotted with anti-BMRP. This same membrane was immunoblotted with anti-Bcl-2 mAb to detect the amount of immunoprecipitated Bcl-2 protein. The amount of total lysate used for immunoprecipitation was 20 times than that used for the Western blot analysis shown in part (A) of the figure. The arrowhead on the **top panel** indicates BMRP protein, and the arrowhead on the **bottom panel** indicates Bcl-2 protein. The BMRP band that co-immunoprecipitates with Bcl-2 corresponds to the upper band shown in the Western blot (A, **upper panel**). Results are representative of three independent experiments. C, matching isotype control antibody; I, anti-Bcl-2 monoclonal antibody. The strong band above the BMRP band corresponds to the light chain of the antibody.

distribution is comparable to that obtained for Bcl-2 (Fig. 3). The distribution of Bcl-2 and BMRP in the various fractions did not appear to change when both proteins were co-expressed in the double transfectant (Fig. 3).

The distribution of mitochondria among the four fractions was assessed by measuring succinate dehydrogenase activity. This enzyme is specific of the mitochondria, and within this organelle, is embedded in the inner membrane. Fractions used in the Western blot analyses of the subcellular localization of the BMRP and Bcl-2 proteins were assayed for succinate dehydrogenase activity. These experiments indicate that approximately 60% of the mitochondria locate to the P1 fraction, and 34% to the HM fraction (Table I). The distribution of mitochondria among the fractions closely resembles that observed for Bcl-2 and BMRP proteins, with high levels in both P1 and HM fractions, and much lower levels in LM and Cy fractions. The succinate dehydrogenase results obtained suggest that a major portion of the Bcl-2 and BMRP proteins that are observed in the P1F is the

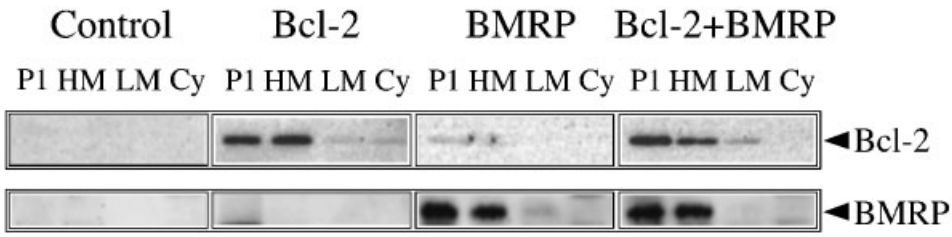

Fig. 3. BMRP and Bcl-2 co-localize to the P1 and heavy membrane fractions of cells. HEK 293T cells were transiently transfected by lipofection with eukaryotic expression vectors producing the indicated proteins. The control was transfected with empty vector. Forty-eight hours after transfection, cell lysates were subjected to subcellular fractionation to separate the P1, HM, LM, and Cy fractions. Pellet fractions were resuspended in buffer and equivalent amounts (by volume) of the four fractions were subjected to SDS–PAGE. **Lower panel**: Immunoblot with anti-BMRP polyclonal antibody. **Upper panel**: Same membranes as in lower panel were stripped and reblotted with anti-Bcl-2 monoclonal antibody. Overexpressed untagged BMRP protein in HEK 293T lysates runs as a single band of approximately 16–17 kDa. Results are representative of three independent experiments.

result of mitochondria that remain in this fraction during the fractionation procedure. Measurement of lactate dehydrogenase activity indicated that the subcellular fractionation procedure utilized in these studies results in very low levels of cytosolic contamination in membranous fractions (3% or less), suggesting that the amounts of Bcl-2 and BMRP proteins in the cytosol are almost negligible (data not shown).

Similar localization results were obtained with the BMRP protein Flag tagged at the carboxyl terminus. In these experiments, the BMRP-C-Flag protein was detected as a doublet and both bands co-located to the same fractions, suggesting that the bottom band is a degradation product generated within the mitochondria (data not shown).

Tissue Distribution Analysis

A riboprobe complementary to the coding region of the *bmrp* mRNA was utilized to assess the tissue distribution of *bmrp* expression by Northern blot analysis. Two multiple tissue Northern blots (MTN^{TM}) containing poly A$^+$ RNA from several human tissues (Clontech) were hybridized with the *bmrp* riboprobe. This analysis revealed relatively broad expression of the *bmrp* gene, with a major *bmrp* mRNA band of approximately 0.8 kb (Fig. 4A,B). In the blot presented in Figure 4A, this band was particularly abundant in the heart and skeletal muscle followed by the liver, pancreas, and kidney. In the second blot (Fig. 4B), testis contains the highest amount of this messenger RNA. Additionally, a larger mRNA of approximately 2.2 kb is observed in some tissues. In the prostate and the brain, this larger mRNA species seems to be as abundant, or slightly more abundant than the smaller 0.8 kb band.

In order to investigate the tissue distribution of the BMRP protein, lysates from several mouse tissues were prepared and the presence of the BMRP protein detected by Western blotting (Fig. 4C). Among the tissues tested, kidney, liver, thymus, and testis present relatively high levels of the BMRP protein, while lower levels are detected in other tissues such as brain and spleen.

TABLE I. Percentage Succinate Dehydrogenase Activity of Subcellular Fractions

	% SDH activity					
Fractions	Control	Bcl-2	BMRP	Bcl-2 + BMRP	AVG	SD
P1	64	55	61	60	60	3.7
HM	33	36	34	35	34.5	1.3
LM	1	4	2	2	2.3	1.3
Cy	2	5	2	4	3.3	1.5

The subcellular fractions from cells transfected with the various constructs were assayed for succinate dehydrogenase activity. Total succinate dehydrogenase activity for each one of the transfected samples was calculated by adding the activities of the four different fractions. A value of 100% was assigned to this total succinate dehydrogenase activity, and percentage succinate dehydrogenase activity for each one of the fractions was calculated. Mean and standard deviation values for each type of fraction are also shown.

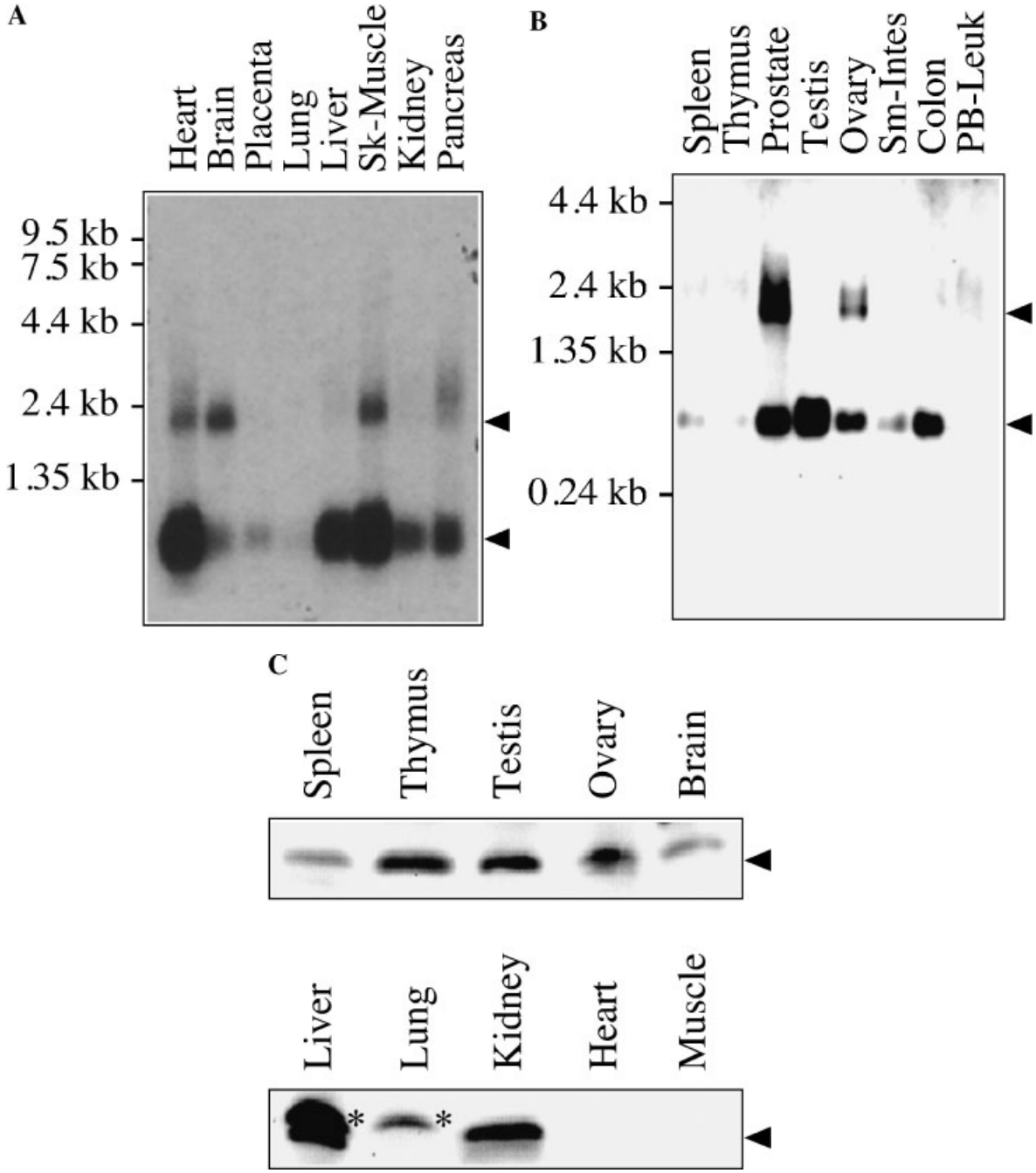

Fig. 4. Tissue distribution of BMRP. **A**: Northern blot analysis (radioactive). Each lane contains approximately 2 μg of poly A^+ RNA from the indicated human tissues. Two specific bands at approximately 0.8 and 2.2 kb are indicated by arrowheads. The location of RNA size markers is also indicated. **B**: Northern blot analysis (non-radioactive) of a poly A^+ RNA blot containing approximately 2 μg of poly A^+ RNA per lane from additional human adult tissues. The colon tissue used contained no mucosa. The two bands at approximately 0.8 and 2.2 kb are indicated by arrowheads. The positions for the RNA size markers are indicated on the left. **C**: Western blot analysis. Total lysates containing 200 μg of total protein from each mouse tissue indicated were loaded per lane. After SDS–PAGE, the proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-BMRP antibody. BMRP protein is indicated by an arrowhead. Muscle denotes skeletal muscle (obtained from mouse leg). The asterisk (*) marks a larger band that could correspond to a posttranslationally modified form of BMRP, or else it could represent a non-specific protein that is recognized by the anti-BMRP serum. Abbreviations: Sk-Muscle, skeletal muscle; Sm-Intes, small intestine; PB-Leuk, peripheral blood leukocyte.

The amounts of *bmrp* mRNA and BMRP protein present in a few of the tissues analyzed do not correlate, muscle tissues being a clear example. This discordance between mRNA and protein levels in some tissues could have been due to species differences, since the Northern blot was performed with human RNA and the Western blot with mouse tissues. An additional Northern blot was conducted with mouse RNA, revealing an expression pattern of mouse *bmrp* mRNA similar to that observed for the human species (data not shown; the major mRNA species observed in this blot is also approximately 0.8 kb). This suggests that the differences observed in the tissue distribution of the *bmrp* mRNA and its encoded protein are not related to the different species utilized (human and mouse) as the source of tissues. It is more likely that the disparities observed are the result of differences in the stability of the *bmrp* mRNA and protein in those tissues, and/or differences in the efficiency of translation of the *bmrp* mRNA. Alternatively, the presence of other highly abundant proteins in some tissues, for example cytoskeletal proteins in the muscle, may result in a lower proportion of BMRP in such tissues.

BMRP Induces Apoptotic Cell Death in Overexpression Studies

The role that BMRP plays in apoptosis was assessed in overexpression studies using transient transfection of mammalian embryonic fibroblasts, as well as other cell lines. In MYS Cl2 BCF1 and HEL 299 cells, transient transfection with expression vectors encoding BMRP induced cell death (Fig. 5A,B). This effect on cell viability caused by BMRP overexpression was similar to that observed by overexpression of Bax or Bad, two well established pro-apoptotic proteins (Fig. 5A,B). BMRP-C-Flag behaved similarly to BMRP in these studies (data not shown). Comparable results to those shown in Figure 5A and B were also obtained in NIH/3T3 cells (Fig. 5C), MEF (mouse embryo fibroblast; data not shown), and HEK 293 (human embryonic kidney; data not shown). Initial studies to determine the role that BMRP plays in apoptosis focused on the establishment of stable clones of the cell line FL5.12 overexpressing the BMRP protein. FL5.12 is an IL-3-dependent prolymphocytic cell line [Boise et al., 1993; Gonzalez-Garcia et al., 1994]. The fact that these attempts failed is consistent with the observation that BMRP causes cell death.

To test whether Bcl-2 is able to protect cells from BMRP induced cell death, Bcl-2 and BMRP proteins were co-expressed in MYS and HEL 299 cells. Bcl-2 increased the survival of cells overexpressing BMRP (Fig. 5A,B), however, the effect of Bcl-2 was only partial since the survival of co-expressing cells did not reach 100% (vector control). This level of protection afforded by Bcl-2 to cells overexpressing BMRP was comparable to that observed for cells that overexpressed pro-apoptotic Bax or Bad proteins (Fig. 5A,B). Baculoviral p35, an inhibitor of caspases, also blocked BMRP-induced cell death. The extent to which p35 protected cells from BMRP-stimulated cell death was similar to that obtained for Bad overexpressing cells (Fig. 5C). These results suggest that BMRP induces apoptosis by a mechanism mediated by caspases, which can be blocked by Bcl-2 or the baculoviral caspase inhibitor p35.

DISCUSSION

In this report, a novel protein that interacts with anti-apoptotic Bcl-2 has been found utilizing the yeast Two-Hybrid system. This protein was identified to be a human mitochondrial ribosomal protein (MRPL41 or MRPL27), and thus it has been referred to throughout this article as BMRP. The interaction between these two proteins observed in the yeast Two-Hybrid assay has been confirmed in mammalian cells utilizing co-immunoprecipitation studies. The subcellular localization of BMRP was determined to assess whether Bcl-2 and BMRP have a common subcellular fractionation pattern. The results obtained indicate that BMRP and Bcl-2 localize to the same fractions, mainly mitochondrial fractions. These data is in accordance with previous studies that showed attachment of Bcl-2 mainly to the outer mitochondrial membrane, and also to other subcellular membranes such as the nuclear envelope [Monaghan et al., 1992; Krajewski et al., 1993]. The intracellular location found for BMRP in these studies is consistent with reports that identify BMRP as one of the ribosomal mitochondrial proteins (MRP-L27 or MRPL41, depending on the report) [Goldschmidt-Reisin et al., 1998; Kenmochi et al., 2001]. Tissue distribution studies revealed the presence of a major *bmrp* mRNA band of approximately 0.8 kb in several

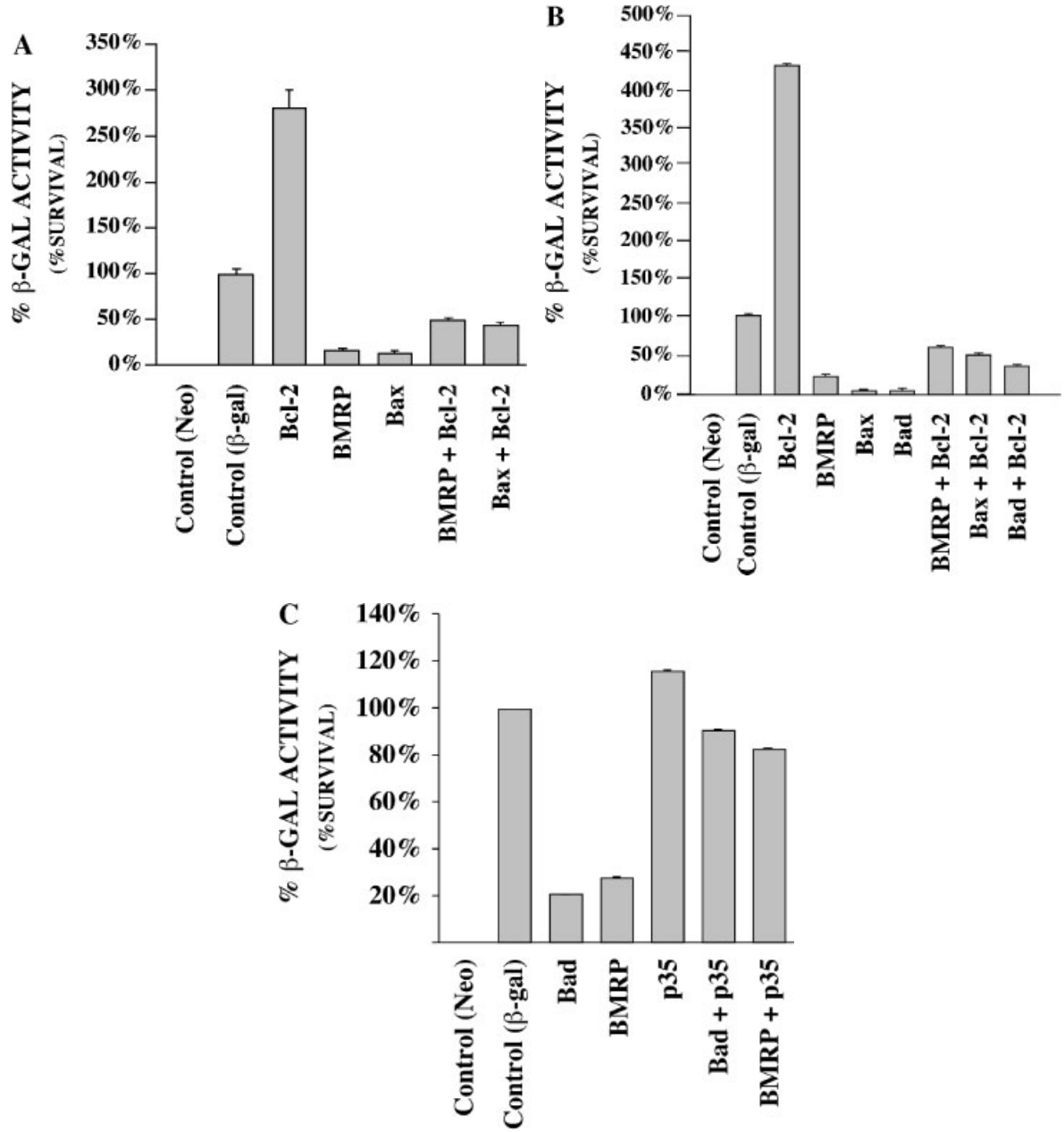

Fig. 5. Overexpression of BMRP induces cell death in mammalian cells, which is counteracted by Bcl-2 and p35. **A**: MYS Cl2 BCF1 cells were transiently transfected by lipofection with eukaryotic expression vectors producing the indicated proteins along with a smaller amount of plasmid encoding β-galactosidase as a reporter. The negative control was transfected with empty vector. 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 five different experiments, each one performed in triplicate. **B**: HEL 299 cells were transiently transfected by lipofection with eukaryotic expression vectors as indicated for MYS cells. Sixty hours after transfection, β-galactosidase activity was determined to estimate the effect on cell survival of overexpression of the various proteins. The experiment shown is representative of three different experiments, each one performed in triplicate. **C**: NIH/3T3 cells were transiently transfected by lipofection essentially as described for MYS and HEL 299 cells. Forty-eight hours after transfection, survival was estimated as in parts (A) and (B). The experiment presented is representative of four different experiments, each one performed in triplicate.

human tissues. Additionally, a larger mRNA species of approximately 2.2 kb is also observed in some tissues. At the RNA level, *bmrp* is particularly abundant in heart, skeletal muscle, liver, testis, and prostate, which is consistent with BMRP being a mitochondrial protein, since most of these tissues contain abundant mitochondria. The distribution of the BMRP protein

as determined by Western blotting with mouse tissues revealed higher amounts of BMRP in liver, kidney, thymus, and testis than in the rest of the tissues tested. Disparities in the amount of mRNA and protein were observed in some tissues. Since Northern blots with human and mouse RNA showed similar expression patterns (suggesting that the disparities are not due to species differences), it is likely that the discordance observed in those tissues is due to differences either in the stability or translation efficiency of the mRNA, or else in the stability or relative abundance of the protein in such tissues.

The role that BMRP plays in apoptosis has been explored in overexpression studies. In these studies, BMRP reduces the viability of transiently overexpressing cells similarly to the effect observed for overexpression of Bax and Bad, two well established pro-apoptotic proteins. These results have been obtained in several human and mouse primary fibroblast cell lines, as well as in the immortalized human and mouse cell lines HEK 293 (data not shown) and NIH/3T3. Additionally, stable clones of the murine cell line FL5.12 could not be established, which is consistent with the observation that BMRP causes cell death. Bcl-2 is an anti-apoptotic protein that has been shown to protect cells from death induced by a variety of apoptotic stimuli [Strasser et al., 1991; Garcia et al., 1992; Reed, 1994]. Bcl-2 was able to counteract the ability of BMRP to kill overexpressing cells. This effect of Bcl-2 on BMRP-induced cell death was comparable to that observed for Bax- and Bad-stimulated cell death. It is likely that the protection against BMRP afforded by Bcl-2 is mediated by the Bcl-2/BMRP protein–protein interaction (Bcl-2 can also interact with Bad and Bax), however, the mechanisms involved have not yet been addressed in the experiments presented. Furthermore, the cell death caused by overexpression of BMRP was prevented by co-expression of the broad-spectrum caspase inhibitor p35. Altogether, these results suggest that BMRP induces cell death by apoptosis.

The mitochondrion is recognized as a key subcellular organelle in the regulation of the apoptotic process. The release of cytochrome c and other apoptogenic factors such as AIF and Smac/Diablo from the mitochondria is a common event in apoptosis induced by a variety of signals. The anti-apoptotic Bcl-2 and Bcl-xL proteins have been shown to inhibit the release of these mitochondrial factors [Kluck et al., 1997; Shimizu et al., 1999]. BMRP has been identified as a Bcl-2 binding protein. It could be speculated that upon receipt of an apoptotic stimulus, BMRP might disassemble from the mitochondrial ribosome and translocate to the cytosol, where it could bind to Bcl-2. Binding of BMRP to Bcl-2 might presumably inhibit the anti-apoptotic activity of this pro-survival factor, inducing the death of the cell by apoptosis. Bcl-2 has been reported to bind to the VDAC component of the PTP [Shimizu et al., 1999]. It has been proposed that the mitochondrial changes leading to the release of apoptogenic factors to the cytosol during apoptosis may be associated with the activity of this channel. BMRP could exert its function by binding and participating in the regulation of the PTP/Bcl-2 mitochondrial complex. An interesting question is whether BMRP triggers apoptosis from within the mitochondrion, or else BMRP is involved in a latent response that is activated after mitochondrial permeabilization, resulting in the release of BMRP and its interaction with Bcl-2. Preliminary observations from overexpression experiments with a construct that expresses Green Fluorescent Protein fused to the amino terminus of WT BMRP (GFP/BMRP), or a BMRP deletion mutant lacking the amino terminal 45 amino acids, BMRP(46–137) (this mutant is devoid of the mitochondrial localization signal), resulted in decreased cell survival, suggesting that BMRP may be able to promote cell death from the cytosol by blocking Bcl-2 (Parsa et al., unpublished observations). However, the localization of these proteins within the cell should be studied, as well as their ability to interact with Bcl-2 and the potency with which they induce cell death. Future experiments will address these hypotheses to try to elucidate the specific role that BMRP plays in apoptosis.

Various cytosolic ribosomal proteins, as well as translation initiation and elongation factors have been found to perform functions other than protein biosynthesis [Wool, 1996]. These unconventional roles include the regulation of cell growth, transformation, and death. Four ribosomal proteins (L13a, L7, S3a, and S29) have been reported to be involved in cell death by apoptosis [Naora et al., 1998; Chen and Ioannou, 1999; Khanna et al., 2000]. Translation initiation factors, such as eIF4G, may play

an important role in the control of cell death through the translation initiation mechanism mediated by internal ribosome entry site elements (IRES) [Holcik et al., 2000]. Recently, two pro-apoptotic proteins have been identified as mitochondrial ribosomal proteins. These two proteins are DAP3 (death-associated protein-3) and PDCD9 (programmed cell death-9) [Kissil et al., 1995; Sun et al., 1998; Carim et al., 1999; Kissil et al., 1999; Cavdar Koc et al., 2001]. PDCD9 is the mammalian homolog of the *Gallus gallus* pro-apoptotic protein p52 [Carim et al., 1999]. The roles that these two proteins play in apoptosis are still unclear. DAP3 was isolated as a mediator of interferon-γ-induced cell death, and has later been shown to participate in TNF-α- and Fas-stimulated apoptosis [Kissil et al., 1995; Kissil et al., 1999]. DAP3 has also been identified as a glucocorticoid receptor (GR) binding protein [Hulkko et al., 2000]. This interaction is ligand-dependent, with a strong interaction occurring only upon ligand binding. Glucocorticoids are strong inducers of apoptosis in thymocytes, however, a role for DAP3 in glucocorticoid-induced apoptosis has not yet been established. BMRP may therefore represent a novel member of a group of mitochondrial ribosomal proteins that are involved in the control of cell death by apoptosis.

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