The Cellular Protein PRA1 Modulates the Anti-Apoptotic Activity of Epstein-Barr Virus BHRF1, a Homologue of Bcl-2, through Direct Interaction

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Running Title: Interaction between BHRF1 and PRA1
SUMMARY

The Epstein-Barr virus-encoded early protein, BHRF1, is a structural and functional homologue of the anti-apoptotic protein, Bcl-2. There is accumulating evidence that BHRF1 protects a variety of cell types from apoptosis induced by various external stimuli. To identify specific proteins from normal epithelial cells that interact with BHRF1 and that might promote or inhibit its anti-apoptotic activity, we screened a yeast two-hybrid cDNA library derived from human normal foreskin keratinocytes and identified a cellular gene encoding human prenylated rab acceptor 1 (hPRA1). The interaction of hPRA1 with BHRF1 was confirmed using GST pull-down assays, confocal laser scanning microscopy and co-immunoprecipitation. Two regions of PRA1, aa 30-53 and the carboxyl-terminal 21 residues, are important for BHRF1 interactions and two regions of BHRF1, aa 1-18 and aa 89-142, including the Bcl-2 homology domains BH4 and BH1, respectively, are crucial for PRA1 interactions. PRA1 expression interferes with the anti-apoptotic activity of BHRF1, though not of Bcl-2. These results indicate that the PRA1 interacts selectively with BHRF1 to reduce its anti-apoptotic activity and might play a role in the impeding completion of virus maturation.

INTRODUCTION

Apoptosis, also called programmed cell death, is an essential and highly conserved and controlled physiological process. It plays an important role in various biological events
including normal development, tissue homeostasis, host defense and suppression of oncogenesis (1, 2). Inappropriate regulation of apoptosis contributes to a wide variety of diseases such as cancer, autoimmune and vascular diseases, acquired immunodeficiency syndrome and neurodegenerative disorders (3-5). Among numerous proteins and genes involved in regulating the evolutionarily conserved apoptotic pathway, the best characterized are the Bcl-2 family of proteins (6, 7), which includes regulatory molecules with both pro- and anti-apoptotic effects. Proteins of the Bcl-2 family possess four conserved Bcl-2 homology (BH)\(^1\) domains, BH1 to BH4, as well as a transmembrane domain (8), and these domains have been shown to mediate protein-protein interactions (8-11). Although the detailed mechanisms of action of the Bcl-2 family proteins remain to be elucidated, it seems that the balance of anti-apoptotic proteins, such as Bcl-2, and pro-cell death proteins, such as Bax, is important in determining the susceptibility of cells to various apoptotic stimuli (12). In addition, several members of the Bcl-2 protein family can interact physically with one another in a complicated network of homo- and heterodimers (11, 13-15), suggesting neutralizing competition between these proteins, whilst other family members have been suggested to regulate apoptosis independently (16). Recently, a number of Bcl-2-binding proteins, which do not belong to this family, have been identified, including Raf-1, calcineurin, Bag-1, R-Ras, H-Ras, p53-BP2, SMN, Bis, BI-1, Beclin and BAR (8, 17-21). Evidence is accumulating that proteins of the Bcl-2 family fulfill their pro- or anti-apoptotic roles largely through exerting their effects on
mitochondria (13, 22). Mitochondria play a pivotal role in the apoptotic signaling pathway by releasing apoptogenic factors from the mitochondrial intermembrane space into the cytoplasm, such as cytochrome c (23) which binds to Apaf-1 and activates caspase-9, thereby eliciting the downstream caspase cascade and inducing apoptosis (13, 22, 24). It has been shown that the anti-apoptotic Bcl-2 family proteins prevent cytochrome c release and in turn prohibit the activation of the caspase cascade and apoptosis (25).

In the face of the tremendous diversity of viruses, higher vertebrates have evolved general defense mechanisms to control viral infection. The initiation of apoptosis by host cells is one such innate host response that can quickly and efficiently remove virus-infected cells, so limiting virus reproduction and total viral burden, reducing or eliminating the dissemination of progeny virus in the host, and minimizing the effects of virus infection and the susceptibility to mutational adaptation by virus (26). Thus, the interaction of specific host cellular proteins with viral proteins that promote proliferation or protect cells from apoptosis is a key determinant in influencing the dynamic equilibrium between life and death and the extent of virus infection. The Epstein-Barr virus (EBV)-encoded anti-apoptotic protein BHRF1 is of particular interest in this regard. EBV is a ubiquitous human gamma herpesvirus which infects B lymphocytes, where it establishes latent infections, and stratified squamous epithelium, where it undergoes productive replication (27) and increases the susceptibility of these cells to oncogenic transformation. EBV is closely associated with a variety of human diseases, including
nasopharyngeal carcinoma (NPC), Burkitt’s lymphoma, gastric carcinoma, Hodgkin’s disease, infectious mononucleosis and lymphoproliferative diseases in immunocompromised hosts, such as acquired immunodeficiency syndrome and transplant recipients (28-31). BHRF1 is expressed during the EBV lytic cycle and is a component of the restricted early antigen complex (32).

The BHRF1 gene is present in the genomes of all natural EBV isolates examined to date and is highly conserved. It shares limited sequence homology to Bcl-2 (33-35); alignment of BHRF1 and Bcl-2 reveals approximately 25% amino acid sequence identity and 42% amino acid sequence similarity (36). Like Bcl-2, BHRF1 is primarily located in the mitochondrial, endoplasmic reticulum (ER) and nuclear membranes (34, 37). Earlier studies indicated that BHRF1 is dispensable for EBV-induced transformation of B lymphocytes and for virus replication in vitro (36, 38). However, previous studies have also demonstrated that BHRF1 protects various cell types from apoptosis induced by a wide range of external stimuli, including growth factor deprivation, serum starvation, ionomycin treatment, heterologous viral infection and DNA-damaging agents, such as cisplatin, etoposide and mitomycin C (39-42). In addition, BHRF1 also protects cells against the apoptotic effects of tumor necrosis factor alpha, anti-Fas antibody, activated monocytes and radiation (34, 39, 43-46). Thus, BHRF1 is both a structural and functional viral homologue of the cellular Bcl-2 protein. This suggests that BHRF1 can inhibit apoptosis during EBV replication, prolonging the life span of EBV-infected
cells and potentiating virus maturation and spread.

In the present study, we explored the cellular factors that modulate the anti-apoptotic activity of BHRF1 through protein-protein interactions. The human prenylated rab acceptor 1 (hPRA1) was identified (47) and its effect on the anti-apoptotic activity of BHRF1 revealed.

EXPERIMENTAL PROCEDURES

Plasmid Construction — A series of expression plasmids were established in vector pBTM116 (48), from which inserted genes were expressed in frame with a LexA DNA-binding domain (LexA-DB). To construct pBTM/NBHRF1, the entire coding sequence of BHRF1, derived from NPC tissue, was subcloned from plasmid pYYS-2 (49). Truncations of NBHRF1, encoding amino acids (aa) 1-88, 1-142, 1-163, 19-191 and 61-191, were generated from pBTM/NBHRF1 by polymerase chain reaction (PCR) amplification of the relevant regions. To construct pBTM/BBHRF1, in which BHRF1 was derived from EBV associated with infectious mononucleosis, an EBV DNA fragment from genome coordinates 54,376 to 54,948 was amplified from cell line B95.8 by PCR. Plasmid pBTM/Bcl-2 contained a full-length human gene, bcl-2, which was subcloned from a Bcl-2-expression plasmid, pCΔj-SV2-bcl-2-neo (a gift from Y. Tsujimoto, Osaka University Graduate School of Medicine, Osaka, Japan) (50). Plasmid pBTM/lamin, containing a lamin gene, was used as a control.

A tetracycline inducible, ‘tet-on’, system (51) was used to express NBHFR1. NBHRF1
was cloned into vector pUHD10.3 and its expression could be turned on by a holo-transactivator, composed of a transactivator and a co-transactivator. The transactivator was expressed from plasmid pUHD172-1neo and the co-transactivator was provided in the form of doxycycline (Dox), a tetracycline derivative.

Vector pcDNA3.1(+) (Invitrogen, Groningen, The Netherlands) was used to express target proteins constitutively. An EBNA-1 tag (aa 408-446 from EBV nuclear antigen-1) from pCF7 (52) was inserted into the vector for convenient identification of the target protein, resulting in the generation of plasmid pcDNA3.1/NA-1. For constitutive expression of BHRF1, wild type (WT) NBHRF1 and a mutant encoding aa 19-191 were inserted into pcDNA3.1/NA-1 in frame with the EBNA-1 tag.

Plasmids expressing rat PRA1 (rPRA1) and polypeptides derived from it were generously provided by J. K. Ngsee (University of Ottawa, Ottawa, Canada) (53). The protein products included WT rPRA1 and polypeptides comprising aa 1-110, 1-164, 30-185 and 54-185, in each case as a fusion protein with a GAL4 activation domain (GAL4-AD) in pGAD424 vector (Clontech, Palo Alto, Calif.) (53). DNA fragments encoding the WT protein and aa 1-164, 30-185 and 54-185 of rPRA1 were subcloned into vector pcDNA3-HA to generate an amino-terminal hemagglutinin (HA)-tag when expressed in mammalian cells. Human PRA1 (hPRA1) plasmid, pGAD-hPRA1, was a gift from C. B. Bruni (University of Napoli, Napoli, Italy) (47) and had the entire gene fused to GAL4-AD in vector pGAD10
To obtain transient expression of hPRA1 in a mammalian cell system, DNA fragments encoding the WT hPRA1 and polypeptides comprising aa 1-164, 30-185 and 54-185 were subcloned into the pcDNA3-HA. Plasmid pGEX-5X-1/NBHRF1 was designed to express a glutathione S-transferase (GST)-BHRF1 fusion protein following subcloning of the NBHRF1 from pYYS-2 into vector pGEX-5X-1 (Amersham Pharmacia, Piscataway, N.J.).

The nucleotide sequences of all constructs were confirmed by direct DNA sequencing.

**Yeast Two-Hybrid Screen**—Yeast two-hybrid screening was carried out as described by Vojtek et al. (54). Briefly, the bait plasmid, pBTM/NBHRF1, and a cDNA library of human normal foreskin keratinocytes (Clontech), in which the cDNA was fused to the GAL4-AD, were used sequentially to transform *Saccharomyces cerevisiae* strain L40. The resulting transformants were then plated on synthetic complete (SC) medium lacking tryptophan (Trp), uracil (Ura), leucine (Leu), lysine (Lys) and histidine (His) (SC-Trp, Ura, Leu, Lys, His) and SC-Trp, Ura, Leu, Lys as a control. His\(^+\) colonies were screened for β-galactosidase activity 3 days later by colony-lift filter assay as described (54). His\(^+\)/LacZ\(^+\) colonies were candidate clones of cDNAs encoding BHRF1-interacting proteins. After subsequent purification and curing processes, the cDNAs were isolated and sequenced using an ABI PRISM\textsuperscript{TM} Dye Terminator Cycle Sequencing Core Kit on the ABI 373A DNA Sequencer (Perkin-Elmer, Foster, Calif.). The resultant DNA sequences were then subjected to a BLAST search in the GenBank data base.
Cell Culture and Transfection—The human embryonic kidney cell line, 293 (55) and RHEK-1 epithelial cells (56) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum plus 100 units/ml penicillin and 100 µg/ml streptomycin. RHEK-1 cells were established by immortalizing primary normal human foreskin keratinocytes with an adenovirus type 12-simian virus 40 hybrid. Transfections were carried out using a modified calcium phosphate method (57). Briefly, plasmid DNA, which was dissolved in 125 mM CaCl₂, 25 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 6.95), 140 mM NaCl and 0.75 mM Na₂HPO₄, was added dropwise to 60% confluent cells and then incubated at 35°C under 3% CO₂ for 18 h. After the medium was refreshed, cells were moved to a 37 °C incubator containing 5% CO₂. For transient expression, cells were harvested 48 h after transfection. To establish stable BHRF1-inducible clones, RHEK-1 cells were co-transfected with pUHD/NBHRF1 and pUHD172-1neo and the transfectants were selected in the presence of 800 µg/ml neomycin (Gibco BRL, Gaithersburg, Md.). A stable BHRF1-inducible clone, N27-2, and a pUHD10.3 vector-transfected control clone, V10-3, were maintained in medium containing 400 µg/ml neomycin.

Specific Antibodies Against BHRF1—Bacterially expressed BHRF1 (49) was used to immunize BALB/c mice and produce monoclonal antibody (mAb). After several booster immunizations, mice with strong and specific immune responses were sacrificed and hybridoma cells were generated as described by Tsai et al. (58). The antibody clone with the
highest reactivity to BHRF1, 3E8, was selected by Western blot analysis and
immunofluorescence assays (manuscript in preparation). BHRF1 was injected subcutaneously
into the backs of rabbits to obtain a specific, polyclonal antiserum. After boosting several times,
specific antisera were determined by Western blot analysis and immunofluorescence assays.

Immunofluorescence Assay—N27-2 cells were grown on glass slides and transfected
separately with HA-tagged hPRA1 or rPRA1. The cells were then induced with 1 µg/ml Dox
for 24 h and fixed with acetone at room temperature for 10 min and the expression of
HA-tagged PRA1 and BHRF1 was detected by double staining. The smear was reacted with
mouse anti-HA mAb (BAbCO, Richmond, Calif.) and rabbit anti-BHRF1 polyclonal
antiserum as primary antibodies for 1 h at 37°C. Secondary antibodies were then applied to
detect the primary antibodies by using Rhodamine Red™-X-conjugated goat anti-mouse
immunoglobulin G (Jackson, West Grove, Pa.) and fluorescein isothiocyanate-conjugated goat
anti-rabbit immunoglobulin G (Jackson). After washing with phosphate-buffered saline, the
cells were mounted in phosphate-buffered saline containing 90% glycerol and examined under
a confocal laser scanning microscope (Axiovert 135M photomicroscope; Zeiss, Oberkochen,
Germany).

Western Blot and Co-immunoprecipitation Assays—To extract proteins from
mammalian cells, the cells were solubilized in lysis buffer (20 mM octyl glucoside, 0.5%
Triton X-100, 0.3 M NaCl, 25 mM sodium phosphate [pH 7.4], 10 µg/ml leupeptin, 10 µg/ml
aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Extracts from yeast were prepared as
described by Sato et al. (14). The cell extracts were then denatured at 100°C for 5 min and
fractionated by SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred
to Hybond™-C super nitrocellulose membrane (Amersham Pharmacia), and the blots were
reacted with specific antibodies as described previously (35). The primary antibodies used in
this study included anti-LexA-DB rabbit polyclonal antiserum (Upstate Biotechnology, Lake
Placid, N.Y.), anti-GAL4-AD mouse mAb (Clontech), anti-GST mouse mAb (kindly provided
by C.-H. Tsai, National Taiwan University, Taipei, Taiwan), anti-HA mouse mAb (BAbCO),
antibHRF1 mouse mAb 3E8, anti-EBNA-1 mouse mAb 5C11 (kindly provided by M.-R.
Chen, National Taiwan University, Taipei, Taiwan) (52), anti-Bcl-2 mouse mAb (clone 124;
Dako, Glostrup, Denmark) and mouse anti-β-actin mAb (clone AC-15; Sigma, St. Louis, Mo.).

For co-immunoprecipitation assay, cells transfected with appropriate expression
plasmids were lysed in lysis buffer, extracts were then incubated with anti-HA mAb or
anti-BHRF1 mAb 3E8 at 4°C overnight. The resulting immunocomplexes were precipitated
with protein A-Sepharose beads (Amersham Pharmacia) at 4°C for 2 h, washed extensively
with Tris-buffered saline, and analyzed by Western blotting.

**GST Pull-Down Assay**—Recombinant GST and GST-BHRF1 fusion proteins were
purified and incubated with glutathione-agarose slurry in binding buffer (25 mM Tris-HCl [pH
7.5], 125 mM NaCl) for 4 h at 4°C. After three washes in binding buffer, the bound GST and
GST-BHRF1 were incubated with in vitro transcription and translation lysates of the

[^{35}S]-methionine-labeled hPRA1 or[^{35}S]-methionine-labeled rPRA1, which was produced

using a TNT coupled reticulocyte lysate system (Promega, Madison, Wis.) in the presence of

[^{35}S]-methionine (NEN, Boston, Mass.). The protein complex bound to the beads was then

eluted with 2X SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 3%

DTT, 0.01 mg/ml bromophenol blue), subjected to SDS- polyacrylamide gel electrophoresis

and analyzed using a PhosphorImager (Storm 840; Molecular Dynamics, Sunnyvale, Ca.).

**Evaluation of Protein Effects on Cell Growth by WST-1 Assay**—N27-2 cells were

transfected transiently with plasmids expressing HA-tagged WT hPRA1, 1-164 hPRA1, and

30-185 hPRA1. One day after transfection, the cells were incubated with 1 µg/ml Dox for 24 h

to induce BHRF1 expression and then trypsinized and plated at 4000 cells per well in 96-well plates. Twenty-four hours after seeding, the cells were treated for 72 h with 0, 50 and 100 µM

cisplatin to induce apoptosis. Cell viabilities were determined by WST-1 assay using cell

proliferation reagent WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) as

specified by the manufacturer. This method is a colorimetric assay to quantitate cell

proliferation and cell viability. The mechanism is based on the cleavage of the tetrazolium salt,

WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate), by

mitochondrial dehydrogenases in viable cells to form highly water-soluble formazan. To study

effects of hPRA1 on Bcl-2, BHRF1 or 19-191 BHRF1, 293 cells were transiently
co-transfected with HA-tagged WT hPRA1 and plasmids expressing Bcl-2, EBNA-1-tagged BHRF1, or 19-191 BHRF1, respectively. The transfectants were plated at 6000 cells per well in 96-well plates and treated for 24 h with 0, 40 and 80 µM cisplatin, and viability of the cells was also determined by WST-1 assays.

RESULTS

**PRA1 Binds to BHRF1** — Previous studies demonstrated that BHRF1 could protect cells from apoptosis triggered by a broad range of stimuli. To identify the cellular factors regulating the anti-apoptotic activity of BHRF1, we employed yeast two-hybrid screening with a cDNA library of human normal foreskin keratinocytes using BHRF1 as bait. Screening of over twenty million yeast transformants enabled the identification of 80 positive clones which interacted specifically and reproducibly with the BHRF1. DNA sequence analysis and BLAST searches of GenBank revealed that one of these clones encodes a DNA fragment homologous to a rat gene encoding prenylated rab acceptor 1 (rPRA1), a protein that associates with Rab and VAMP2 (53). Recently, a human PRA1 (hPRA1) gene was identified by Bucci et al. (47) and its published cDNA sequence is identical to our BHRF1-interactive clone.

To verify the specificity of the BHRF1-PRA1 interaction, full-length hPRA1 or rPRA1 was fused to GAL4-AD and subsequently co-transformed into L40 cells with the bait plasmid expressing LexA-DB tagged-NBHRF1. Protein expression of individual clones was confirmed by Western blotting using specific antibodies as shown in figure 1A-1C. The two-hybrid assays
showed that all transformants could grow on SC-Trp, Ura, Leu, Lys plate (His\textsuperscript{+} plate). However, the reporter genes, \textit{HIS3} and \textit{LacZ}, were activated only when NBHRF1 and either hPRA1 or rPRA1 were co-expressed in the yeast cells, as demonstrated by growth of colonies on SC-Trp, Ura, Leu, Lys, His plate (His\textsuperscript{−} plate) and development of blue coloration in the colony-lift filter \(\beta\)-galactosidase assay. This interaction-dependent activation of \textit{HIS3} and \textit{LacZ} was not observed when NBHRF1 was expressed alone or when hPRA1 or rPRA1 was co-expressed with lamin as negative controls. The same results were also obtained when BHRF1 of B95.8 origin (BBHRF1) was applied (Fig. 1D). Thus, these data demonstrate that both hPRA1 and rPRA1 can interact specifically with BHRF1.

To substantiate the interaction of PRA1 and BHRF1, a GST pull-down assay was carried out. The amount of purified GST and GST-BHRF1 fusion proteins and \textit{in vitro} translated \(^{35}\text{S}\)-methionine-labeled hPRA1 and rPRA1 subjected for this assay are shown in figure 2A and 2B, respectively. The GST-BHRF1 fusion protein, but not GST protein, could precipitate the hPRA1 or rPRA1 protein from the translation products (Fig. 2C), indicating that BHRF1 directly binds PRA1 \textit{in vitro}. These results are consistent with those of the yeast two-hybrid assays.

To determine whether both PRA1 and BHRF1 co-localize in cells, the BHRF1-inducible clone (N27-2) was transfected along with HA-tagged rPRA1 or hPRA1 and treated with Dox to induce the expression of BHRF1 transiently. PRA1 was detected using mouse monoclonal
anti-HA antibody and BHRF1 was visualized with the specific rabbit antiserum. The results were analyzed by confocal laser scanning microscopy and are shown in figure 3. Distribution of either rPRA1 or hPRA1 was found to co-localize with BHRF1 in the transfected cells, with a perinuclear pattern of cytoplasmic staining.

Evidence of cytoplasmic co-localization supports the hypothesis that these two proteins may be associated in vivo. We carried out a co-immunoprecipitaion assay to provide further evidence that BHRF1 is associated with PRA1 in vivo. The clone N27-2 and a vector control, clone V10-3 (data not shown), were transiently transfected, separately, with HA-tagged rPRA1, hPRA1, or control plasmid pcDNA3-HA. The transfectants were then incubated with or without 1μg/ml Dox for 24 h. Cell extracts prepared from the Dox-treated or untreated cells were separately subjected to immunoprecipitation with either anti-HA antibody or an anti-BHRF1 antibody and the resulting immunocomplexes analyzed subsequently by Western blotting. The results are shown in figure 4. BHRF1 could be precipitated from PRA1-transfected N27-2 cells, induced with Dox, using anti-HA antibody (Fig. 4B, lanes 6 and 10). This interaction was also detected in a reciprocal co-immunoprecipitation assay (Fig. 4B, lanes 8 and 12). Conversely, the HA-tagged PRA1 was immunoprecipitated only with anti-HA antibody but not with anti-BHRF1 antibody from PRA1-transfected N27-2 cells without Dox induction (which expressed only HA-tagged PRA1) and BHRF1 could not be detected in these immunocomplexes (Fig. 4B, lanes 5, 7, 9 and 11). Protein expression by each transfectant also
was confirmed by Western blotting as illustrated in figure 4A. Additionally, similar results also were observed in another mammalian cell line, 293 cells, which were co-transfected with plasmids expressing the BHRF1 and HA-tagged PRA1 (data not shown). These data indicate that BHRF1 interacts specifically with PRA1 in mammalian cells.

**Identification of Domains in PRA1 which Associate with BHRF1**—In order to define the regions of PRA1 that interact with BHRF1, we studied a series of truncated rPRA1 proteins using two-hybrid assays. Protein expression by each rPRA1 mutant was determined by Western blotting with anti-GAL4-AD antibody (Fig. 5A). The results of the two-hybrid assays showed that only the WT rPRA1 and mutant 30-185 rPRA1, which comprised aa 30 to 185, enabled the growth of the L40 cells on SC-Trp, Ura, Leu, Lys, His plate (Fig. 5C) and formed a blue color in the colony-lift filter β-galactosidase assay (Fig. 5D). This suggests that the first 29 aa of rPRA1 are not required for association with BHRF1. Mutants 1-110 rPRA1, 1-164 rPRA1, and 54-185 rPRA1 did not support cell growth (Fig. 5B-5C). In parallel, BHRF1 of B95.8 origin (BBHRF1) was applied to this assay and also found to associate with WT rPRA1 and 30-185 rPRA1 (data not shown). The observations indicate that aa 30 to 53 and the last 21 residues at the carboxyl-terminus of rPRA1 are important for interactions with BHRF1.

To further support this notion, we performed co-immunoprecipitation assays with HA-tagged hPRA1 truncation mutants. The HA-tagged hPRA1 mutants were constructed and transfected transiently into the N27-2 cells with or without Dox treatment. Protein expression
by each HA-tagged hPRA1 mutant and Dox-induced BHRF1 are illustrated in figure 6A, and the immunoprecipitation results are shown in figure 6B. As expected, only HA-tagged WT hPRA1 and deletion mutant 30-185 hPRA1 could be co-immunoprecipitated with BHRF1 using anti-BHRF1 antibody (Fig. 6B, lanes 8 and 16), HA-tagged 1-164 hPRA1 and 54-185 hPRA1 were not precipitated in these assays (Fig. 6B, lanes 12 and 20). In the reciprocal experiments, BHRF1 could be co-precipitated with HA-tagged WT hPRA1 or 30-185 hPRA1 (Fig. 6B, lanes 6 and 14), but not 1-164 hPRA1 or 54-185 hPRA1 (Fig. 6B, lanes 10 and 18), when anti-HA antibody was used for immunoprecipitation. BHRF1 was not detected following immunoprecipitation from cells expressing the HA-tagged hPRA1 alone, using anti-HA or anti-BHRF1 antibody (Fig. 6B, lanes 5, 7, 9, 11, 13, 15, 17 and 19). Similar results also were obtained from another mammalian cell line, 293 cells, following transient co-transfection with the BHRF1 plasmid and the plasmids encoding the HA-tagged hPRA1 mutants (data not shown). In summary, the results from the co-immunoprecipitation experiments were consistent with those of rPRA1 from the yeast two-hybrid assays and showed the regions of PRA1 spanning aa 30 to 53 and the last 21 aa at the carboxyl-terminus are crucial for associations with BHRF1.

Identification of Domains in BHRF1 which Associate with PRA1—To identify the regions of the BHRF1 required for interactions with PRA1, a series of deletion mutants of BHRF1 were cloned with LexA-DB and their interactions with rPRA1 were assessed using
two-hybrid assays. Expression of individual BHRF1 mutants was confirmed using immunoblot assays with the anti-LexA-DB antibody (Fig. 7B). The WT BHRF1, mutants 1-163 BHRF1 and 1-142 BHRF1, which lack of the carboxyl-terminal 28 and 49 aa, respectively (Fig. 7A), were able to interact with rPRA1 in these assays. In contrast, 19-191 BHRF1 (deletion of the first 18 aa), 61-191 BHRF1 (deletion of the first 60 aa) and 1-88 BHRF1 (deleted from aa 89 to 191) failed to interact with rPRA1. These results suggest that the last 49 aa at the carboxyl-terminus of BHRF1, including the BH2 and transmembrane domains, were not required for associations with rPRA1. On the other hand, the first 18 aa (including the BH4 domain) and aa 89 to 142 (including the BH1 domain) of BHRF1 were critical for interactions with PRA1.

*The Interaction Between PRA1 and BHRF1 Interferes the Anti-apoptotic Activity of BHRF1*—To gain insight into the functional significance of the BHRF1-PRA1 interaction, we investigated the effect of PRA1 on the anti-apoptotic activity of BHRF1. The clone N27-2 was transiently transfected with HA-tagged WT hPRA1, 1-164 hPRA1, 30-185 hPRA1 plasmids, or the vector control, separately. After growth with or without Dox induction, the transfected cells were incubated with cisplatin, a DNA-damaging agent, to cause cell death. Viable cells were detected by WST-1 assays and the relative viability of each set of transfected cells was compared to the control, which was transfected with HA-tagged vector and was not treated with Dox and cisplatin. Protein expression of each transfectant was confirmed by Western blot.
analysis (Fig. 8A). In the presence of cisplatin, the HA-tagged vector-transfected cells showed the higher survival rate if BHRF1 expression was induced (Fig. 8B, lanes 9-10, 17-18). These results are consistent with a previous study showing that BHRF1 conferred resistance to cisplatin-induced cell death (34, 41, 43). Cells transfected with 1-164 hPRA1, which did not interact with BHRF1, exhibited comparable viability in the presence of BHRF1 expression (Fig. 8B, lanes 14 and 22). However, the survival rates of cells containing WT hPRA1 or 30-185 hPRA1, both of which showed interactions with BHRF1, even in the presence of BHRF1 expression, fell off to a degree comparable to the transfectants that did not express BHRF1 (Fig. 8B, lanes 12 and 20; 16 and 24). Similar results were obtained when apoptosis was induced by another DNA-damaging agent, mitomycin C (data not shown). Taken together, our findings provide a nice correlation between BHRF1-PRA1 interactions and reduction of BHRF1’s anti-apoptotic activity.

PRA1 Does Not Interact with Bcl-2 and Exerts No Impact on the Anti-apoptotic Activity of Bcl-2—Since BHRF1 is a viral homologue of Bcl-2, structural similarity between BHRF1 and Bcl-2 leads us to address whether Bcl-2 also interacts with PRA1. Bcl-2 was also cloned in frame with LexA-DB and tested for interaction with GAL4-AD tagged-PRA1 in the yeast two-hybrid assays (Table I). In contrast to the reactivity shown by BHRF1, Bcl-2 was not found to associate with either hPRA1 or rPRA1 and behaved like the negative control, lamin. Thus, although Bcl-2 is structurally and functionally similar to BHRF1, it did not interact with
PRA1 in two-hybrid assays.

To determine whether PRA1 has any effect on the anti-apoptotic activity of Bcl-2, 293 cells were transiently co-transfected with plasmids expressing Bcl-2 and HA-tagged WT hPRA1. In parallel, cells were also co-transfected transiently with plasmids expressing HA-tagged WT hPRA1 and EBNA-1-tagged WT BHRF1. Since the first 18 aa of BHRF1 were required for interaction with PRA1, the mutant EBNA-1-tagged 19-191 BHRF1 was included for comparison, as well as the vector control. Protein expression of each transfectant was analyzed by Western blotting (Fig. 9A). The transfected cells were then treated with cisplatin to induce apoptosis and assessed for cell viability. As shown in figure 9B, in the presence of cisplatin, cells expressing BHRF1 alone revealed a higher survival rate than cells containing an EBNA-1-tagged vector or expressing WT hPRA1 only (Fig. 9B, lanes 9-11, 17-19). Consistent with above findings, overexpressed WT hPRA1 reduced the cell viability conferred by BHRF1 to a similar level to the negative control (Fig. 9B, lanes 12 and 20). However, Bcl-2 and 19-191 BHRF1 displayed anti-apoptotic activity and showed similar viabilities to the wild type BHRF1 but the presence of WT hPRA1 did not interfere with their ability to prevent cell death (Fig. 9B, lanes 13-16, 21-24). These data demonstrate that PRA1 did not affect the anti-apoptotic activity of Bcl-2, consistent with the lack of a physical interaction.
DISCUSSION

In this study, we identified a cellular protein, PRA1, which interacts specifically with EBV BHRF1 in a yeast two-hybrid system. This interaction also could be demonstrated using a GST-BHRF1 fusion protein in GST pull-down assays. Evidence of a physical association at the cellular level was provided by reciprocal co-immunoprecipitation of BHRF1 and PRA1 and co-localization in RHEK-1 and 293 cells using confocal laser scanning microscopy assays. The PRA1 protein is highly conserved, rPRA1 shares 95% amino acid identity with hPRA1 (47), and shows similarity to a yeast gene product, Yip3p (53). In this study, we also demonstrated that both rPRA1 and hPRA1 are able to interact specifically with BHRF1 in the yeast two-hybrid system, in biochemical assays in vitro and in co-immunoprecipitation experiments.

The exact physiological function of PRA1 is still largely unknown. PRA1 was originally identified using a yeast two-hybrid screen, as a protein that associates with prenylated Rab GTPases and synaptic vesicle protein VAMP2, both of which are involved in intracellular vesicle transport, but not with other small Ras-like GTPases such as the Rho/Rac family. The deduced amino acid sequence predicts that PRA1 contains two extensive hydrophobic domains which are both predicted to form β sheets and may form a membrane-spanning domain or the inner hydrophobic core of the protein. Immunofluorescence assays showed that PRA1 is preferentially localized to the Golgi apparatus (59). PRA1 is expressed ubiquitously but is more abundant in the placenta, pituitary gland, kidney and stomach tissues (47, 53). A recent
study demonstrated that PRA1 inhibits the removal of Rab from the membrane by GDP
dissociation inhibitor (59). Binding of PRA1 to VAMP2 can be displaced by Rab3A,
suggesting that Rab and VAMP2 may share a common binding site in PRA1, and that PRA1
may play a role in the control of vesicle docking and fusion (53, 59).

Deletion analysis of PRA1, using two-hybrid and co-immunoprecipitation assays,
indicates that the regions of PRA1 spanning aa 30-53 and the last 21 aa at the
carboxyl-terminus are important for BHRF1 interaction or for protein conformation. A
remarkable feature of the amino acids 30-53 region is the abundance of arginine residues,
whilst the extreme carboxyl-terminal 21 amino acids of PRA1 is rich in charged acidic
 glutamate residues. The BHRF1-interacting domains of PRA1 are the same as those which
interact with Rab3A and VAMP2 (53) and it is possible that BHRF1 may compete with these
two proteins for binding to PRA1. Whether the BHRF1 affects the function of Rab3A and
VAMP2 and vesicular transport pathways remains to be addressed. We also cannot rule out the
possibility that BHRF1 may also exert regulatory effects on PRA1. The biological significance
of the interaction between BHRF1 and PRA1 needs to be explored further.

In addition, PRA1 contains one or more potential phosphorylation sites clustered around
residues 35-50 (53), within the BHRF1-interacting domain, aa 30-53. This may imply that
phosphorylation or dephosphorylation of PRA1 plays a critical role in BHRF1 binding.
Furthermore, functional studies showed that both WT PRA1 and 30-185 PRA1, which
associated physically with BHRF1, decreased its anti-apoptotic activity, but these two proteins themselves did not promote apoptosis. However, the 1-164 PRA1 mutant was defective in BHRF1 binding and also failed to reduce the anti-apoptotic activity of BHRF1. These results strongly imply that repression of the anti-apoptotic activity of BHRF1 is dependent on the physical interaction with PRA1.

Mapping of the PRA1 binding domain(s) of BHRF1 showed that the first 18 aa (including the BH4 domain) and aa 89-142 (including the BH1 domain) were critical for interaction with PRA1. Previous studies also indicated that the BH1 domain is required for Bcl-2 and Bcl-XL to interact with Bax (11, 60) and the BH4 domain for Bcl-2 to interact with Raf-1 (61). Thus, the BH1 and BH4 domains are also important for Bcl-2 interaction with other Bcl-2 family homologues and with other cellular proteins. However, in contrast to BHRF1, Bcl-2 did not interact with PRA1 in yeast two-hybrid assays. Functional analysis revealed that PRA1 did not associate with Bcl-2 and 19-191 BHRF1 (which lacks the first 18 aa, including the BH4 domain) and had no effect on their death repressor activities. These data suggest that the lack of physical interaction between PRA1 and Bcl-2 and 19-191 BHRF1 means that it does not affect their anti-apoptotic activity. Although BHRF1 belongs to Bcl-2 family, it shares only distant sequence homology (about 25%) with human Bcl-2, that similarity residing mainly in the BH1 and BH2 domains and at the carboxyl-terminal end. Comparison with Bcl-2 over the amino-terminal domains BH4 and BH3 reveals poor
homology. Moreover, BHRF1 has a much shorter loop region between the BH4 and BH3
domains than Bcl-2, with no significant amino acid similarity between the loop regions,
suggesting the possibility of different functions for these regions (62). Another disparity
between BHRF1 and Bcl-2 is that Bcl-2 is phosphorylated in vivo and this modification has
been demonstrated to be important in regulating Bcl-2 function (63, 64). BHRF1 has been
predicted to possess two potential N-glycosylation sites (32). Thus, differences in amino acid
sequence and post-translational modification could potentially explain the differential abilities
of BHRF1 and Bcl-2 to interact with cellular PRA1.

It has been suggested that Bcl-2 family proteins exert their pro- or anti-apoptotic activity
mainly through acting on mitochondria (22). Previous studies showed that cellular protein
14-3-3 bound to phosphorylated Bad, a pro-apoptotic Bcl-2 homologue, and prevented it from
moving to the mitochondria, thereby inhibiting apoptosis mediated by Bad (65). Another Bcl-2
family protein, death agonist Bim, binds to LC8 dynein light chain, and is sequestered by the
microtubule-associated dynein motor complex, preventing translocation to mitochondria, and
eventually thwarting its pro-apoptotic activity (66). Zhu et al. stressed the importance of
correct protein localization for Bcl-2 function using Bcl-2 mutants targeted to different
subcellular organelle membranes (67). Recently, a cellular reticulon family protein, RTN-xS,
was found to interact with both Bcl-XL and Bcl-2 and change their subcellular localization
from the mitochondria to the ER, leading to a reduction in their anti-apoptotic activities (68).
Based on these results, we postulate that cellular protein PRA1, which is predominantly located in the Golgi apparatus, interacts with BHRF1, but not Bcl-2, and may sequester BHRF1 from the mitochondria to the Golgi apparatus, thereby hampering its anti-apoptotic activity. However, the precise molecular mechanisms underlying the blockage of anti-apoptotic activity need to be clarified further.

To our knowledge, this is the first report indicating that a non-Bcl-2 family protein PRA1 specifically and selectively interacts with viral Bcl-2 homologue BHRF1, but not with Bcl-2, and reduces the anti-apoptotic activity of BHRF1. The molecular mechanism of BHRF1 in anti-apoptosis is still obscure. Our findings imply that selective interaction of BHRF1 with a cellular target protein, not targeted by Bcl-2, may have evolved to regulate the dynamic balance between agonists and antagonists of apoptosis. Given that the activities of BHRF1 and Bcl-2 are regulated by different mechanisms, such as various binding proteins with apparently distinct functions, BHRF1 probably acts through alternate regulatory mechanism and may function differently from Bcl-2. It is also conceivable that BHRF1 could associate with other cellular proteins (our unpublished data), and the mechanisms underlying the effects of PRA1 and the other proteins on BHRF1 activity might be totally different.

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FOOTNOTES

1 The abbreviations used are: BH, Bcl-2 homology domain; EBV, Epstein-Barr virus; NPC,
nasopharyngeal carcinoma; ER, endoplasmic reticulum; h (r) PRA1, human (rat) prenylated rab acceptor 1; VAMP2, vesicle associated membrane protein 2; aa, amino acids; LexA-DB, LexA DNA-binding domain; GAL4-AD, GAL4 activation domain; PCR, polymerase chain reaction; Dox, doxycycline; WT, wild type; HA, hemagglutinin; GST, glutathione S-transferase; SC, synthetic complete medium; mAb, monoclonal antibody.


FIGURE LEGENDS

FIG. 1. Interaction of BHRF1 with PRA1 in the yeast two-hybrid system. Lysates from L40 yeast cells containing pBTM116 vector expressing the LexA DNA-binding domain (LexA-DB) or plasmids expressing LexA-DB-tagged BHRF1 fusion proteins (NBHRF1, BBHRF1) were analyzed by Western blotting using anti-LexA-DB antibody (A) or anti-BHRF1 antibody (B). Cells containing vector expressing GAL4 activation domain (GAL4-AD), or plasmids expressing GAL4-AD-tagged rat PRA1 (rPRA1) or human PRA1 (hPRA1) fusion proteins, were probed with anti-GAL4-AD antibody (C). Interaction of BHRF1 with PRA1 is shown by the activation of the HIS3 and LacZ reporters (D). L40 yeast cells expressing NBHRF1, BBHRF1 or Lamin with rPRA1, hPRA1 or GAL4-AD were tested for growth by plating on a SC-Trp, Ura, Leu, Lys plate (His$^+$ plate) (left) and a SC-Trp, Ura, Leu, Lys, His plate (His$^-$ plate) (middle) and for β-galactosidase activity (β-Gal) (right) using colony-lift filter assay as described in Materials and Methods. Molecular weight markers are indicated in kilodaltons. NBHRF1, BHRF1 of nasopharyngeal carcinoma origin; BBHRF1, BHRF1 of B95.8 origin.

FIG. 2. Interaction of BHRF1 with PRA1 in GST pull-down assays. Western blots of purified GST and GST-BHRF1 fusion proteins were probed with anti-GST antibody (top) and anti-BHRF1 antibody (bottom) (A). In vitro-translated, $[^{35}S]$-methionine-labeled rPRA1 and hPRA1 proteins were analyzed by SDS-polyacrylamide gel electrophoresis and PhosphorImaging (B). -, in vitro translation without DNA template as a negative control.
Purified GST and GST-BHRF1 fusion proteins immobilized on glutathione-agarose were incubated with \[^{[35}S\]-methionine-labeled rPRA1 or hPRA1 separately. After washing, labeled proteins were eluted from the beads and analyzed by SDS-polyacrylamide gel electrophoresis and PhosphorImaging (C). Molecular weight markers are indicated in kilodaltons.

**FIG. 3. Co-localization of BHRF1 and HA-tagged PRA1.** A stable clone, N27-2 cells, was transiently transfected with HA-tagged rPRA1 (A to C) or hPRA1 (D to F) and induced to express BHRF1 by treatment with doxycycline. The cells were reacted primarily with polyclonal rabbit anti-BHRF1 serum (A and D) and monoclonal mouse anti-HA antibody (B and E) and secondarily with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody and Rhodamine Red™-X-conjugated goat anti-mouse antibody and observed using confocal laser scanning microscopy. Panel C is panel A merged with panel B; panel F is panel D merged with panel E.

**FIG. 4. Co-immunoprecipitation of BHRF1 and PRA1 in vivo.** A stable clone (N27-2), expressed BHRF1 after induction with doxycycline (Dox), was transfected transiently with plasmids encoding HA-tagged hPRA1 or HA-tagged rPRA1. Expression of target proteins by the cells, in the presence (+) or absence (-) of 1µg/ml Dox, was examined by Western blot analysis (A). HA-tagged hPRA1 and HA-tagged rPRA1 were detected by anti-HA antibody
BHRF1 (middle) and an internal control, β-actin (bottom), were detected by specific antibodies as indicated. Proteins were immunoprecipitated (IP) from cell extracts by anti-HA antibody (HA) and anti-BHRF1 antibody (BH), respectively. The immunocomplexes were fractionated by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting with anti-HA (top) and anti-BHRF1 (bottom) antibodies (B). Molecular weight markers are indicated in kilodaltons.

FIG. 5. Mapping of the BHRF1-interaction domain of PRA1 using the two-hybrid system.

Wild type (WT) rPRA1 and its truncated mutants were cloned into vectors as GAL4 activation domain (GAL4-AD) fusion constructs (A, top). The co-ordinates of the mutants and their predicted hydrophobic domains (hatched boxes) are indicated by the corresponding amino acids (aa) in the intact molecule. All the constructs were introduced into L40 yeast cells which contained a bait plasmid expressing LexA DNA-binding domain (LexA-DB)-tagged BHRF1. Expression of each rPRA1 fusion protein was detected by Western blotting using anti-GAL4-AD antibody (A, bottom). All the transformants were plated on a SC-Trp, Ura, Leu, Lys plate (B) and a SC-Trp, Ura, Leu, Lys, His plate (C) to test cell growth, and assessed for β-galactosidase activity by colony-lift filter assay (D). Plate sections: 1, WT rPRA1; 2, 1-110 rPRA1; 3, 1-164 rPRA1; 4, 30-185 rPRA1; 5, 54-185 rPRA1. Molecular weight markers are indicated in kilodaltons.
Fig. 6. **Co-immunoprecipitation of BHRF1 and hPRA1 mutants.** The N27-2 clone was transfected transiently with HA-tagged wild type (WT) hPRA1, 1-164 hPRA1, 30-185 hPRA1, 54-185 hPRA1, or vector control. Cells were induced with (+) or without (-) 1μg/ml doxycycline (Dox) and verified by Western blot analysis (A). HA-tagged hPRA1 products (top), BHRF1 (middle), and internal control β-actin (bottom) were detected by specific antibodies as indicated. Protein extracts from the cells were immunoprecipitated (IP) by anti-HA antibody (HA) and anti-BHRF1 antibody (BH). The immunocomplexes were fractionated by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting with anti-HA (top) and anti-BHRF1 (bottom) antibodies (B). Molecular weight markers are indicated in kilodaltons.

Fig. 7. **Mapping of PRA1-interaction domain of the BHRF1.** The wild type (WT) and deletion mutants of BHRF1 were cloned in plasmid pBTM116 (A) and expressed as LexA DNA-binding domain (LexA-DB) tagged fusion proteins in L40 yeast cells. The protein products were recognized by anti-LexA-DB antibody in Western blot analysis (B). Interaction between PRA1 and each BHRF1 mutant was determined by two-hybrid assays as described in figure 1 and the results are indicated in panel A. +, positive reaction; -, negative reaction; BH1 to BH4, Bcl-2 homology domains 1 to 4; TM, transmembrane domain; aa, amino acids. Molecular weight markers are indicated in kilodaltons.
FIG. 8. **Effects of PRA1 on BHRF1 anti-apoptotic function.** The N27-2 cells were transiently transfected with plasmids expressing HA-tagged wild type (WT) hPRA1, 1-164 hPRA1, 30-185 hPRA1 or vector control. The cells were induced with (+) or without (-) 1µg/ml doxycycline (Dox). Western blot analysis was employed to confirm the expression of the HA-tagged PRA1 proteins by anti-HA antibody (A, top), BHRF1 by anti-BHRF1 antibody, (A, middle), and the internal control β-actin by specific antibody (A, bottom). The cells were then treated with 0, 50 and 100 µM cisplatin to induce apoptosis. After 72 h, cell viability was determined by WST-1 assay (B). The percentage of viable cells was calculated relative to vector-transfected N27-2 cells, which were not treated with Dox and cisplatin. Data are presented as the mean ± standard deviation of three independent experiments, and each experiment was performed in triplicate. Molecular weight markers are indicated in kilodaltons.

FIG. 9. **Effects of PRA1 on the anti-apoptotic activities of BHRF1 and Bcl-2.** 293 cells were transiently co-transfected with plasmids expressing Bcl-2, EBNA-1-tagged wild type (WT) BHRF1, or 19-191 BHRF1 together with HA-tagged WT hPRA1. Target proteins from cell lysates were analyzed by Western blotting with anti-HA, anti-EBNA-1 or anti-Bcl-2 antibodies. An internal control, β-actin, was also probed with specific antibody (A). The transfected cells were treated with cisplatin at the indicated concentrations for 24 h and then assessed for cell
viability as described in figure 8B (B). Data are presented as the mean ± standard deviation of three separate experiments performed in triplicate. Molecular weight markers are indicated in kilodaltons.
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+, positive reaction; -, negative reaction; ND, not done
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Fig. 9, Long-Yuan Li et al.
The cellular protein PRA1 modulates the anti-apoptotic activity of Epstein-Barr virus BHRF1, a homologue of Bcl-2, through direction interaction
Long-Yuan Li, Hsiu-Ming Shih, Mei-Ying Liu and Jen-Yang Chen

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