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The Ability of BHRF1 To Inhibit Apoptosis Is Dependent on Stimulus and Cell Type

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The development of resistance to host defense mechanisms such as tumor necrosis factor (TNF)- and Fas-mediated apoptosis of transformed or virus-infected cells may be a critical component in the development of disease. To find genes that protect cells from apoptosis, we used an expression cloning strategy and identified BHRF1, an Epstein-Barr virus (EBV) early-lytic-cycle protein with distant homology to Bcl-2, as an anti-apoptosis protein. Expression of BHRF1 in MCF-Fas cells conferred nearly complete resistance against both anti-Fas antibody and TNF-mediated apoptosis. In addition, BHRF1 protected these cells from monocyte-mediated killing but failed to protect them from killing mediated by lymphokine-activated killer cells. The ability of BHRF1 to protect MCF-Fas cells from apoptosis induced by various stimuli was identical to that of Bcl-2 and Bcl-x_L. Moreover, the mechanism of action of BHRF1 resembled that of Bcl-2 and Bcl-x_L as it inhibited TNF- and anti-Fas-induced activation of two enzymes participating in the apoptosis pathway, cytosolic phospholipase A_2 and caspase-3/CPP32, but did not interfere with the activation of NF-κB-like transcription factors. A putative function of BHRF1 in EBV-infected epithelial cells may be to protect virus-infected cells from TNF- and/or anti-Fas-induced cell death in order to maximize virus production. Surprisingly, expression of neither BHRF1 nor Bcl-2 in a B-cell line, BJAB, protected the cells from anti-Fas-mediated apoptosis even though they increased the survival of serum-starved cells. Thus, the protective role of BHRF1 against apoptosis resembles that of Bcl-2 in being cell type specific and dependent on the apoptotic stimulus.

Tumor necrosis factor (TNF) is a multifunctional cytokine capable of inducing apoptosis in a number of transformed or virus-infected cells (10, 18, 44). The pleiotropic effects of TNF are mediated by two distinct but structurally related cell surface receptors, TNF-R1 and TNF-R2 (47, 51). TNF receptors are members of a larger family of related membrane proteins which are defined by the presence of extracellular cysteine-rich repeats (40). Two members of this so-called TNF receptor superfamily, TNF-R1 and Fas (APO1/CD95), also share a region of intracellular homology designated the "death domain" (15, 46). This domain is an essential component of the signal pathway that triggers apoptosis. Fas ligand (FasL) is expressed predominantly in activated T cells and shows strong cytotoxic activity against Fas-expressing cells (43). FasL, like TNF, has antiviral activity and is also involved in the downregulation of immune responses (31).

Like other apoptotic stimuli, signaling from TNF and FasL converges into a common cell death pathway (for a review, see reference 30). This common pathway is tightly regulated by intracellular decision points (34). One is constituted by the Bcl-2 family of proteins, which is comprised of positive and negative regulators of cell death (for a review, see reference 57). The different members share three Bcl-2 homology domains (BH1, BH2, and BH3) which enable the proteins to homo- or heterodimerize, and the ratio of death antagonists (Bcl-2, Bcl-x_L, Mcl-1, and A1) to death agonists (Bax, Bak, Bcl-x_S, and Bad) dictates whether a cell will respond to apoptotic stimuli (57).

The new and growing family of caspases, cysteine proteases related to interleukin-1β-converting enzyme (ICE/caspase-1), are activated in apoptosis and are now recognized as major effectors of apoptotic cell death (for a review, see reference 33). The importance of caspases in TNF- and Fas-mediated apoptosis has been clearly demonstrated by studies showing that (i) receptor activation leads to proteolytic activation of at least three family members, FLICE/MACH/caspase-8, CPP32/caspase-3, and ICE-LAP3/caspase-7 (8, 29, 37); (ii) inhibitors of caspases effectively block TNF- and Fas-mediated apoptosis (9, 24, 48); and finally, (iii) ectopic expression of caspases in several cells results in apoptosis (27, 55, 59). One of the substrates for caspases is poly(ADP-ribose) polymerase (PARP), which is involved in DNA repair (49). It is cleaved and inactivated by CPP32/caspase-3 during apoptosis (49). Recently, TNF was shown to induce caspase-dependent cleavage and activation of cytosolic phospholipase A_2 ($cPLA_2$) (58). As inhibitors of $cPLA_2$ partially inhibit TNF-induced apoptosis without inhibition of caspase activity, $cPLA_2$ is likely to be a mediator of TNF-induced cell death acting downstream of the caspases (58). The activity of $cPLA_2$ has also been shown to be crucial for TNF-induced lysis of adenovirus-infected cells (52).

Viruses have evolved different strategies affecting different parts of the apoptotic pathway in order to avoid apoptosis (for a review, see reference 38). For example, establishment of an effective adenoviral infection depends on the function of the 19-kDa E1B protein, which shows distant homology to Bcl-2 and is able to protect cells from TNF- and Fas-mediated apoptosis (56). Other examples are the p35 protein of baculovirus and CrmA of cowpox virus, which are able to bind and inhibit multiple members of the caspase family and thereby to prevent apoptosis induced by various stimuli (3, 4, 9, 48).

In this study, using a cDNA expression cloning strategy, we have identified BHRF1, an Epstein-Barr virus (EBV) early lytic protein with sequence homology to Bcl-2, as an anti-apoptosis protein. Expression of BHRF1 in MCF-Fas cells renders them nearly completely resistant against both anti-Fas- and TNF-mediated apoptosis. However, our data also show that expression of BHRF1 in a B-cell line, BJAB, does not

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protect them from anti-Fas-mediated apoptosis, suggesting that the protective role of BHRF1 is cell type dependent, like that of Bcl-2. Furthermore, we show that the TNF- and anti-Fas-induced activation of $cPLA_2$ and caspase-3/CPP32 is inhibited in BHRF1-expressing MCF-Fas cells, whereas TNF-induced NF-κB-activation is not affected. A putative role of BHRF1 in virus infection is discussed.

MATERIALS AND METHODS

Cell lines, culture conditions, and treatments. The MCF7S1 cell line is a subclone of MCF7S breast carcinoma cells selected by limiting dilution of MCF7S cells for high sensitivity to recombinant human TNF (17). The Fas-sensitive MCF-Fas subclone was obtained by transfecting a Fas receptor expression construct into the MCF7S cells (17). MCF-Bcl-2.1 and MCF-Bcl-2.2 are two pooled populations of MCF-Fas cells successfully transfected with a pEBS7 expression construct coding for human Bcl-2. MCF-Bcl-x.1 and MCF-Bcl-x.2 are two pooled populations of the same cells successfully transfected with a similar expression construct coding for human Bcl-x_L, and MCF-V.1 and MCF-V.2 are pools of MCF-Fas cells transfected with an empty vector (17). The Fas-sensitive BJAB cells, a B-cell line, were kindly provided by F. Wang (Harvard Medical School, Cambridge, Mass.). All cells were cultured at 37°C in a humidified air atmosphere with 5% CO_2. RPMI 1640 medium, with L-alanyl-L-glutamine (Gibco Ltd., Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone), streptomycin (10 mg/ml), and penicillin (100 U/ml), was used as a growth medium for all cell lines and is referred to as complete medium (CM). The medium used for the culture of cell lines transfected with either pREP4- or pEBS7-based plasmids was supplemented with hygromycin (Calbiotech, La Jolla, Calif.): 0.15 mg/ml for MCF7S1 and MCF-Fas cells and 0.5 mg/ml for BJAB cells. The growth medium for MCF-Fas cells was further supplemented with 0.2 mg of G418 (Gibco) per ml. Recombinant human TNF was from Genentech, San Francisco, Calif., and monoclonal anti-Fas antibody (anti-APO1 [54]) was kindly provided by P. Krammer (German Cancer Research Center, Heidelberg, Germany).

Plasmids. pREP4 (Invitrogen) is an episomal eucaryotic expression vector in which the presence of both the EBV origin for plasmid replication (*oriP*) and the gene encoding Epstein-Barr nuclear antigen 1 allow the vector to replicate extrachromosomally. The plasmid contains a gene coding for hygromycin phosphotransferase, which confers resistance to hygromycin B. Inserted cDNA in a multicloning site is under the transcriptional control of the Rous sarcoma virus 3′ long terminal repeat. pRP-BHRF1 is the pREP4 plasmid containing cDNA coding for BHRF1. pRP-βgal is the pREP4 plasmid containing cDNA coding for the *Escherichia coli* enzyme β-galactosidase. pEBS7 is also an EBV-based episomal vector, which differs from pREP4 only by having a cytomegalovirus promoter (17). pEBS7-Bcl-2 is the pEBS7 plasmid containing the cDNA coding for human Bcl-2 (17).

Functional expression, cloning, and sequencing. A poly(T)-primed cDNA library (kindly provided by M. Buchwald, University of Toronto, Toronto, Ontario, Canada [42]) made from mRNA from TNF-resistant EBV-transformed lymphoblasts cloned into vector pREP4 was used in the expression study. The cDNA library consisted of 5×10^6 individual colonies, and gel electrophoresis of released inserts revealed a continuous DNA smear ranging in size from approximately 300 to 4,000 bp. The cDNA library was introduced to the highly TNF-sensitive MCF7S1 cells by electroporation; 4×10^8 cells were transfected with 20 to 60 μg of library DNA ml^{-1}, resuspended in CM, transferred to tissue culture flasks, and incubated at 37°C. Two days later hygromycin (150 U/ml) was added, and the incubation was continued for 5 to 8 days to allow drug-resistant clones to reach confluence. To obtain TNF-resistant clones, hygromycin-resistant clones were treated with 50 to 100 ng of TNF per ml for 24 h and incubated in CM containing hygromycin (150 U/ml) until they reached subconfluence. Thereafter, the cells were trypsinized and plated on petri dishes, and treatment with TNF was repeated. When the cells reached confluence following the second TNF treatment, episomal plasmid DNA was recovered by Hirt extraction (14) and introduced into DH10B electrocompetent bacteria (Gibco) by electroporation, as recommended by the manufacturer. Transformants were plated on Luria-Bertani agar-ampicillin (120 μg/ml) plates and incubated for 16 h at 37°C. Colonies were resuspended in Luria-Bertani medium (by using a rubber policeman) and incubated in a bacterial shaker for 24 h at 37°C, and large-scale plasmid DNA was prepared by standard methods. DNA sequences were determined by T7 DNA polymerase (Sequenase, version 2.0; U.S. Biochemicals, Cleveland, Ohio) following the protocol suggested by the manufacturer.

Electroporation. Circular plasmid DNA was introduced into cells by electroporation; 2×10^7 to 4×10^7 cells in logarithmic growth were trypsinized, resuspended in 0.8 ml of phosphate-buffered saline (PBS) containing 20 to 60 μg of plasmid DNA, incubated at room temperature for 10 min, and transferred to an electroporation cuvette. Following delivery of an electric pulse (capacitance, 960 μF; voltage, 850 V/cm; resulting time constant, 11 to 13 ms), cells were incubated in the cuvette at room temperature for 5 min before resuspension in 20 ml of CM and transfer into 75-cm^2 tissue culture flasks. After 48 h at 37°C, hygromycin was added and the incubation was continued until drug-resistant clones reached confluence.

Western blot analysis. Cells, 1×10^6 to 3×10^6 per lane, lysed in Laemmli sample buffer (23) or urea buffer followed by sonication, were resolved in a sodium dodecyl sulfate–12% polyacrylamide gel and transferred to Hybond ECL filter paper (Amersham International, Buckinghamshire, United Kingdom) with an electroblotter (Millipore). Filters were blocked by incubation with 5% low-fat milk powder in Tris-buffered saline (TBS) at 25°C for 1 h and washed twice in TBS containing 0.05% Tween 20 (washing buffer). The filters were incubated for 1 h at 25°C or for 18 h at 4°C with primary antibodies diluted in the following concentrations in washing buffer containing 0.5% low-fat milk powder: anti-BHRF1, from Advanced Biotechnologies Inc., 1:4,000; anti-Bcl-2, kindly provided by G. Nunez, 1:500; and anti-PARP, kindly provided by G. G. Poirier, 1:10,000. Following three washes, the filters were incubated for 30 min at 25°C in washing buffer containing a 1:40,000 dilution of peroxidase-conjugated secondary antibody (Dako Immunoglobulins, Glostrup, Denmark) and 5% low-fat milk powder. After the filters were washed thoroughly, the chemiluminescence reaction was performed and they were exposed to ECL hyperfilm according to the manufacturer's instructions (Amersham).

Cell viability assay. The microculture tetrazolium assay was used to measure the viability of cells (28). Briefly, approximately 5,000 (MCF7S1 or MCF-Fas) or 20,000 (BJAB) target cells per well were plated in 96-well microtiter plates in 0.1 ml of CM, in medium containing the indicated amounts (see the legend to Fig. 2) of TNF and anti-Fas antibody together with 1 μg of protein A (Sigma Chemical Co., St. Louis, Mo.) per ml, or in RPMI supplemented with the indicated concentrations of FCS. After the indicated times (see the legend to Fig. 2) at 37°C, 25 μl of dimethylthiazolyl tetrazolium solution (MTT) (5 mg/ml; Sigma) was added and the incubation was continued for 3 h before addition of 100 μl of lysis buffer (20% sodium dodecyl sulfate in a 50% solution of *N*,*N*-dimethylformamide [pH 4.5]). Forming formazan crystals were allowed to dissolve for 16 h before the plates were read on a microplate reader, using a wavelength of 550 nm.

Effector cells. Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood obtained from healthy donors at the University Hospital of Copenhagen (Rigshospitalet). Sixty milliliters of blood was mixed and resuspended in 60 ml of PBS. PBMC were obtained by centrifugation of erythrocytes through a Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) layer and collection of cells in the interphase. The cells were washed two times in PBS, resuspended in CM, and incubated on petri dishes for 1 h at 37°C at a concentration of 1×10^5 to $3 \times 10^5/cm^2$ in order to isolate the monocytes; the monocytes adhered to the surface while the rest of the mononuclear cells rested in solution. To obtain lymphokine-activated killer (LAK) cells, the nonadherent cells were resuspended at a concentration of 1×10^6 to 2×10^6 cells/ml in CM supplemented with interleukin-2 (300 U/ml; Amersham) and incubated for 3 to 5 days at 37°C. To obtain monocytes, the adherent cells were collected with a rubber policeman, resuspended in 4 ml of CM and centrifuged through a Percoll layer consisting of 43% Percoll (Pharmacia) and 57% CM. The cell layer in the interphase was collected, washed, and resuspended at a concentration of 10^6 cells/ml in CM supplemented with *E. coli* lipopolysaccharide (0.5 μg/ml; Sigma) and incubated at 37°C for 1 h.

Cytotoxicity assay. Target cells (1×10^6 to 2×10^6 cells/ml) were labeled with sodium [^{51}Cr]chromate (0.1 mCi/ml; NEN, Boston, Mass.) in CM for 1 h and washed twice in PBS. The assay was set up in triplicate in 96-well microtiter plates (Greiner). Target cell suspension, 5,000 cells in 0.1 ml of CM, was added to each well with 0.1-ml dilutions of effector cells, TNF, or anti-Fas antibody in CM. After incubation for the required time at 37°C, ^{51}Cr released into 0.1 ml of supernatant from each well was determined with an automatic gamma counter.

Assay for phospholipase A_2 activity. The incorporation of [^{3}H]arachidonic acid into membrane phospholipids of intact cells was done essentially as described previously (16). Briefly, 5×10^6 cells in 5 ml of CM supplemented with [5,6,8,9,11,12,14,15-*N*-^{3}H]arachidonic acid (0.3 mCi/ml; NEN) were plated in 10-cm-diameter petri dishes. After 16 h at 37°C, cells were scraped with a rubber policeman, washed three times with PBS, and resuspended in CM at a concentration of 4×10^5/ml. The assay was set up in triplicate in 24-well microtiter plates by adding 0.5 ml of cell suspension to each well with 0.1 ml of CM or medium containing the indicated dilutions (see Fig. 5) of TNF or anti-Fas antibody. After 18 h at 37°C, the plates were centrifuged at $1,000 \times g$ and 0.3 ml of the supernatant was collected, mixed with 3 ml of Ecosint A scintillation fluid (National Diagnostics, Manville, N.J.), and analyzed in a scintillation counter. The radioactivity of ^{3}H released from cells treated with TNF or anti-Fas antibody was compared with the average radioactivity of ^{3}H released from cells incubated with medium alone and was expressed as a percentage, with the spontaneous value being 100%. The spontaneous release of arachidonic acid from untreated cells was always less than 10% of the total.

Transient transfection and luciferase assay. Five micrograms of plasmid pBIIx, containing NF-κB binding sequences from human immunodeficiency virus, or pAP-LUC, containing an AP-1 binding site from the human collagenase gene upstream from the luciferase reporter gene, was cotransfected with 3 μg of plasmid pEBS-βGal into 2×10^6 to 5×10^6 cells, essentially as described previously (18, 22, 36). Two days after transfection, the cells were left untreated or were treated with the indicated concentrations (see Fig. 4) of TNF 4 h before harvesting. Luciferase activity was measured with the Promega luciferase assay

system. The lysates used for the luciferase assay were also tested for β-galactosidase activity (control for transfection efficiency) by using *ortho*-nitrophenyl-β-D-galactopyranoside (Sigma) as a chromogenic substrate, and the values from the luciferase assay were corrected accordingly.

RESULTS

Cloning of BHRF1 by functional expression cloning. To identify proteins that protect cells from the cytotoxic effects of TNF, a cDNA library originating from TNF-resistant EBV-transformed lymphoblasts was transfected into MCF7S1 cells by electroporation. Hygromycin-resistant cells were exposed to 50 ng of TNF per ml for 24 h, and surviving cells (0.1 to 1%) were allowed to reach subconfluence before another 24-h exposure to TNF. Episomal plasmid DNA from surviving cells was recovered by HIRT extraction (14) and amplified in bacteria; the selection process was repeated three times. The resistance of transfected cells to TNF clearly increased by each round, and a plasmid containing a cDNA insert of approximately 1.7 kb was enriched. After three selection rounds, the 1.7-kb insert was found in approximately 5% of the plasmids. The rescued plasmid containing the 1.7-kb insert was transfected into MCF7S1 cells, and hygromycin-resistant cells were tested for their sensitivity to TNF in a 48-h MTT cell viability assay (28). The sensitivity was compared with that of hygromycin B-resistant MCF7S1 cells transfected in parallel with an empty control vector, pEBS7. The 1.7-kb insert clearly protected cells from the TNF-induced cytotoxicity, as it conferred 80 to 90% resistance to TNF killing at concentrations of up to 100 ng/ml (data not shown). The cDNA was then sequenced, and comparison of the obtained sequence with the GenBank database revealed nearly complete homology (99%) to published cDNA sequences encoding BHRF1. BHRF1 is an EBV early lytic protein with a molecular mass of 17 kDa. BHRF1 displays limited homology to Bcl-2, with the alignment of BHRF1 and Bcl-2 revealing approximately 25% amino acid identity.

BHRF1 protects MCF-Fas cells from TNF- and Fas-mediated apoptosis. To allow a comparison of TNF- and Fas-induced killing in the same cell line, MCF7 cells successfully transfected with human Fas cDNA (MCF-Fas) were used (17). To test the ability of BHRF1 to protect MCF-Fas cells from anti-Fas- and TNF-mediated apoptosis, cells were transfected with pREP4 expression vector containing the cDNA encoding BHRF1 (pRP-BHRF1). The cells were, in parallel, transfected with the pREP4 vector containing cDNA coding for β-galactosidase or with a closely related pEBS7 expression plasmid (empty vector). Two independent transfections of pRP-BHRF1 generated two hygromycin B-resistant cell populations (MCF-BHRF1.1 and MCF-BHRF1.2) that stably expressed BHRF1, as analyzed by Western blotting (Fig. 1).

The sensitivity of transfected MCF-Fas cells to TNF- and anti-Fas-mediated apoptosis was analyzed in a 48-h MTT cell viability assay. As shown in Fig. 2, the control cells (MCF-pEBS7 and MCF-βgal) were killed in a dose-dependent manner. In contrast, MCF-Fas cells expressing BHRF1 were nearly completely resistant to apoptosis mediated by either TNF or anti-Fas. The degree of resistance conferred by BHRF1 was identical to that obtained by overexpression of either Bcl-2 or Bcl-x_L in MCF-Fas cells (17). The protective effect of BHRF1 was emphasized by the morphological changes of control cells versus BHRF1-expressing cells after stimulation with TNF or anti-Fas. As shown in Fig. 2c, untreated MCF-pEBS7 and MCF-BHRF1.1 cells grew equally well and were morphologically alike, whereas the difference was clear for cells treated with TNF. After 24 h with TNF, control cells displayed morphological alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detached from the dish, whereas BHRF1-expressing cells did not show any morphological changes. The same pattern of morphological changes was seen following treatment with anti-Fas (data not shown).

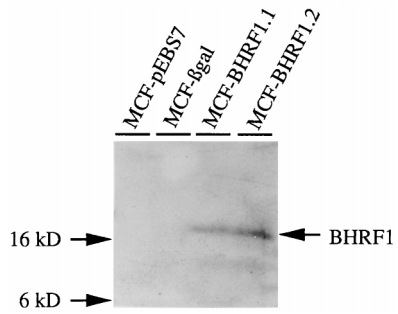

FIG. 1. Detection of BHRF1 expression in transfected MCF-Fas cells by Western blot analysis. MCF-Fas cells were transfected with pRP-BHRF1 (MCF-BHRF1.1 and MCF-BHRF1.2), pRP-βgal (MCF-βgal), or pEBS7 (MCF-pEBS7) by electroporation. Cell lysates from approximately 3×10^6 hygromycin-resistant cells were analyzed by Western blot analysis employing anti-BHRF1 antibody (Advanced Biotechnologies Inc.) and ECL reagents (Amersham). Molecular mass markers are indicated on the left.

BHRF1 protects MCF-Fas cells from cytotoxicity mediated by monocytes and serum starvation but not from LAK cells. To test whether BHRF1 protects cells from mediators of cell death other than TNF or anti-Fas, the killing of cells by activated monocytes, LAK cells, and serum starvation were tested. The sensitivity of transfected MCF-Fas cells to killing by activated monocytes was analyzed by an 18-h chromium release assay with MCF-Fas transfectants as target cells and activated monocytes as effector cells. Control cells were killed in a dose-dependent manner ranging from 3 to 38% at effector-to-target ratios ranging from 1:1 to 64:1 (Fig. 3a). BHRF1 protected cells from killing by monocytes in a manner similar to that in which cells were protected from killing by TNF (Fig. 3a and 2a).

A 4-h chromium release assay with LAK cells as effector cells and MCF-Fas transfectants as target cells showed that BHRF1 was unable to protect cells from LAK-cell-mediated killing: 7 to 90% of the target cells were killed in 4 h at effector-to-target cell ratios ranging from 1:1 to 64:1. There was no significant difference in killing of cells expressing BHRF1 and vector-transfected cells (Fig. 3a). Similar results were obtained with MCF-Fas cells overexpressing either Bcl-2 or Bcl-x_L, both proteins conferring resistance against monocyte-mediated cytotoxicity but failing to protect cells against LAK-cell-mediated killing (Fig. 3b).

To test whether BHRF1 was able to protect cells from serum starvation, the survival of transfected MCF-Fas cells in low serum concentrations (0.5 to 2%) was tested by a 7-day MTT assay. The survival of BHRF1-expressing cells was approximately twice as high as that of vector-transfected control cells (Fig. 3c). Thus, the ability of BHRF1 to protect MCF-Fas cells from serum starvation is of a magnitude similar to that of Bcl-2 or Bcl-x_L (18).

BHRF1 does not interfere with activation of NF-κB-like and AP-1 transcription factors. TNF, but not anti-Fas, activates NF-κB-like and AP-1-like transcription factors in MCF-Fas cells (17). In order to analyze if the expression of BHRF1 interferes with the activation of these two transcription factors, a luciferase reporter gene assay was used employing MCF-Fas cells transiently transfected with a plasmid containing two κB

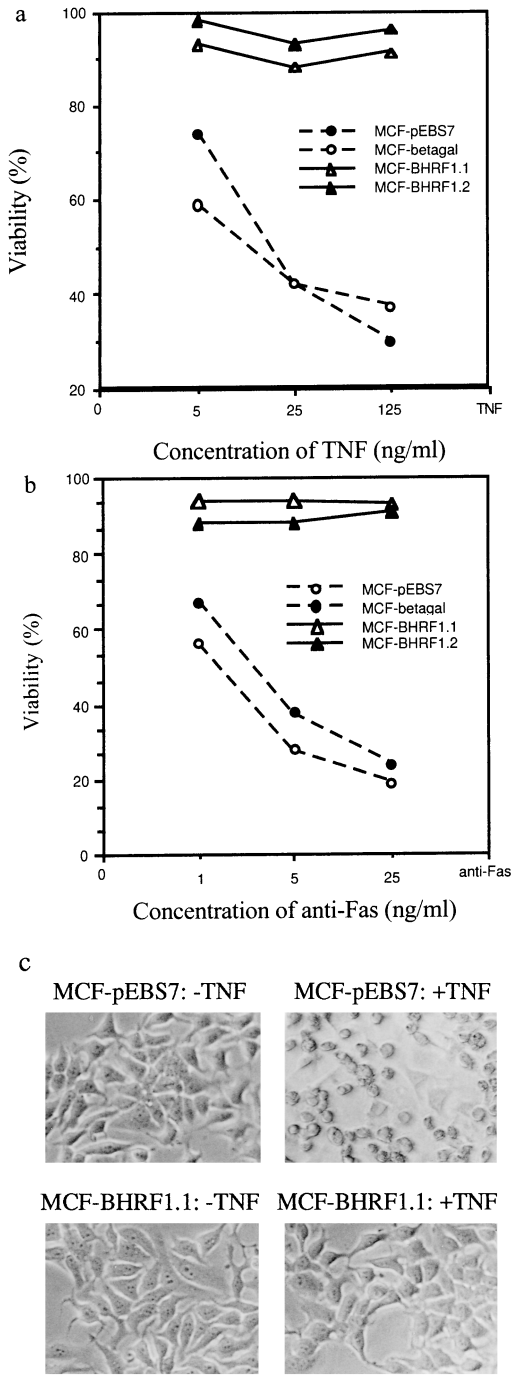

FIG. 2. BHRF1 protects MCF-Fas cells from TNF-mediated (a, c) and anti-Fas antibody-mediated (b) apoptosis. Approximately 5,000 cells per well were plated in 96-well plates with the indicated concentrations of TNF or anti-Fas antibody with 1 μg of protein A per ml, and the percentage of surviving cells was analyzed by MTT assay after a 2-day incubation (a and b). Cell pools are as described for Fig. 1. To show the morphological features of MCF-Fas transfectants treated with 50 ng of TNF per ml for 24 h, approximately 200,000 cells per well were plated in 24-well plates in CM or CM containing TNF (c).

binding motifs (pBIIX) or a human AP-1 binding motif (pAP-LUC) and a minimal mouse *fos* promoter upstream from the firefly luciferase gene (17, 18, 36). Exposure of vector-transfected cells to 10 ng of TNF per ml for 4 h resulted in a 100-fold increase in NF-κB-driven luciferase activity and a

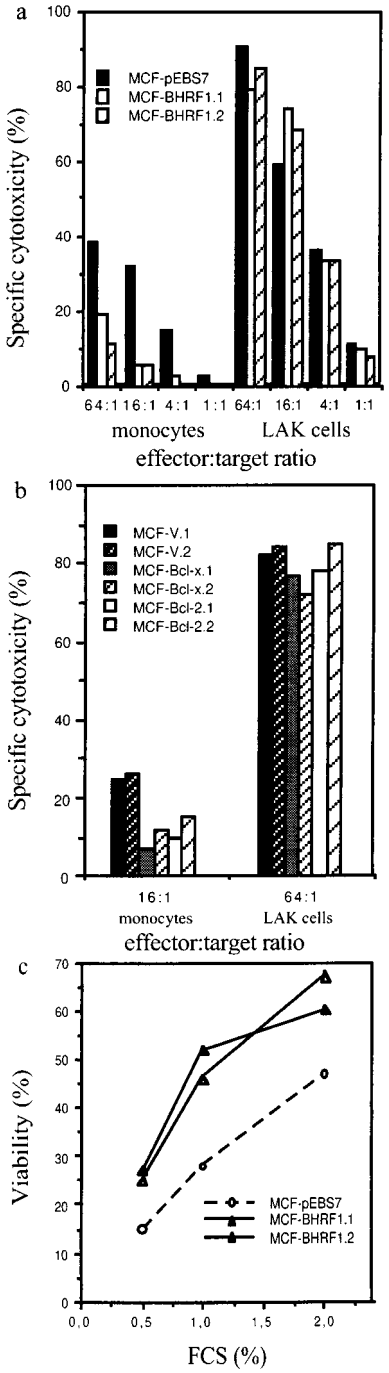

FIG. 3. Effect of BHRF1, Bcl-2, or Bcl-x_L on the sensitivity of MCF-Fas cells to monocytes, LAK cells, and serum starvation. The sensitivity of transfected MCF-Fas cells to lipopolysaccharide-stimulated monocytes or LAK cells was analyzed by chromium release assay. Approximately 5,000 cells labeled with sodium [^{51}Cr]chromate were plated in 96-well plates with the indicated effector-to-target cell ratios for 18 h in the monocyte assay and 4 h in the LAK cell assay (a and b). To test the sensitivity of cells to serum starvation, approximately 5,000 cells per well were plated in 96-well plates with the indicated concentrations of FCS, and the percentage of surviving cells was analyzed by MTT assay after a 7-day incubation (c). Experiments were repeated three times with essentially the same results.

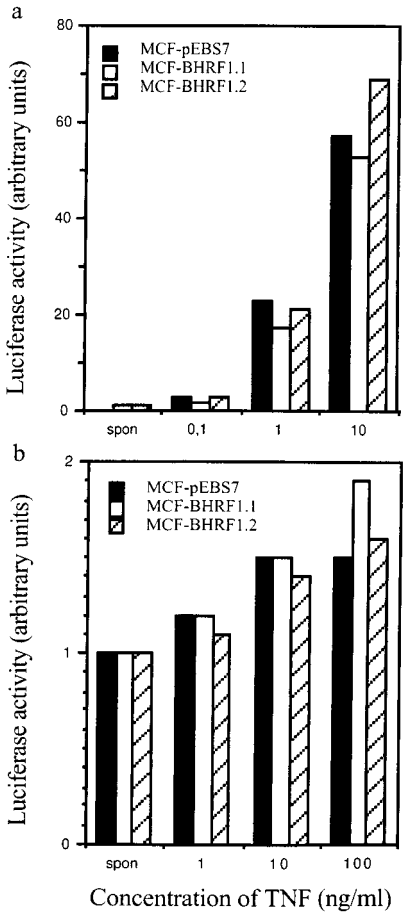

FIG. 4. BHRF1 does not affect TNF-induced activation of NF-κB-like (a) and AP-1 (b) transcription factors. Luciferase activity was measured in cells transiently transfected with plasmid pBIIX, containing two copies of NF-κB binding sites (a), or pAP-LUC, containing an AP-1 binding site upstream of the *Photinus pyralis* luciferase coding sequence (b). The efficiency of each transfection was monitored by a cotransfected cytomegalovirus promoter-driven LacZ construct (pEBS7-βgal). Forty-eight hours after transfection, cells were either left unstimulated or stimulated for 4 h with the indicated concentrations of TNF before harvesting. The lysates were tested for luciferase activity and β-galactosidase activity.

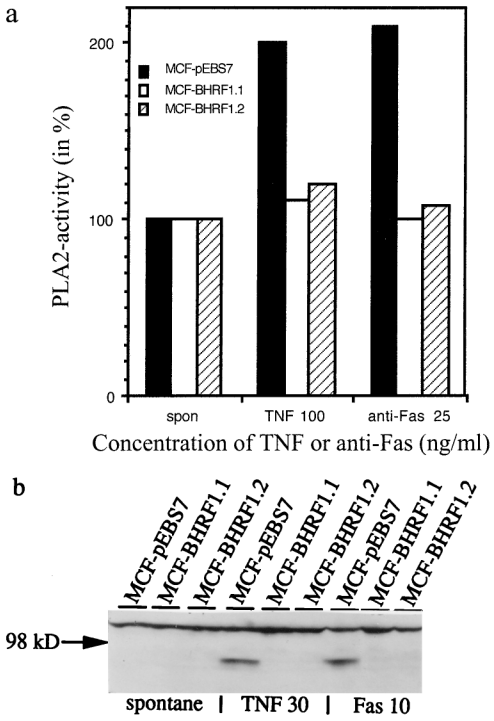

FIG. 5. BHRF1 inhibits the activation of $cPLA_2$ (a) and caspase-3 (b) induced by either TNF or anti-Fas in MCF-Fas cells. $cPLA_2$ activity was measured in cells labeled with [^{3}H]arachidonic acid. The assay was set up in triplicate in 24-well microtiter plates by adding 2×10^5 cells in 0.6 ml of medium or medium containing the indicated concentrations of TNF or anti-Fas plus protein A; after 18 h of incubation at 37°C, 0.3 ml of the supernatant was collected and the radioactivity released was analyzed in a scintillation counter. The spontaneous release was set to 100%. In the PARP analysis, MCF-Fas transfectants were exposed to TNF (30 ng/ml) or anti-Fas (10 ng/ml) for 24 h, after which cell lysates were prepared for immunoblotting with the anti-PARP monoclonal antibody as described in Materials and Methods. The upper band represents the intact protein, and the lower band denotes the signature cleavage product. The migration of a 98-kDa molecular mass marker is indicated on the left.

1.5-fold increase in AP-1-driven luciferase activity (Fig. 4). The expression of BHRF1 did not affect the activation of either NF-κB-like or AP-1-like transcription factors (Fig. 4). Similar results have been obtained with MCF-Fas cells overexpressing Bcl-2 or Bcl-x_L (17).

Activation of $cPLA_2$ by TNF and anti-Fas is inhibited by BHRF1. The release of arachidonic acid from membrane phospholipids by $cPLA_2$ has been suggested to be an essential link in TNF- and anti-Fas-mediated cytotoxicity and has been shown to be inhibited by overexpression of Bcl-2 or Bcl-x_L (17). To test whether BHRF1 was able to inhibit TNF- or anti-Fas-induced activation of $cPLA_2$, the release of radioactivity from cells labeled with [^{3}H]arachidonic acid was measured. An 18-h exposure of vector-transfected MCF-Fas cells (MCF-pEBS7) to TNF (1 to 100 ng/ml) or anti-Fas (1 to 25 ng/ml) induced a dose-dependent release of radioactivity, the maximum release being 2.0-fold (TNF) or 2.1-fold (anti-Fas) higher than the release from cells incubated in medium alone. The TNF- or anti-Fas-induced activation of $cPLA_2$ in MCF-Fas cells expressing BHRF1 was almost completely inhibited when compared to the vector-transfected control cells (Fig. 5a). The level of inhibition by BHRF1 is comparable to that of Bcl-2 or Bcl-x_L in MCF-Fas cells (17).

BHRF1 inhibits the activation of caspase-3. To see whether BHRF1 was able to inhibit the TNF- and anti-Fas-induced activation of CPP32/caspase-3, the integrity of the death substrate PARP was analyzed. Vector-transfected MCF-Fas cells revealed partial cleavage of the native 116-kDa PARP to the signature 85-kDa proteolytic fragment within a 24-h exposure to TNF (30 ng/ml) or anti-Fas (10 ng/ml). In MCF-Fas cells expressing BHRF1 there were no detectable levels of the 85-kDa proteolytic product, indicating that BHRF1 inhibits the activation of CPP32/caspase-3-like proteases (Fig. 5b). Similarly, TNF and anti-Fas failed to induce cleavage of PARP to the 85-kDa fragment in MCF-Fas cells overexpressing either Bcl-2 or Bcl-x_L (data not shown).

BHRF1 does not protect BJAB cells from Fas-mediated apoptosis. As shown above, BHRF1 is able to protect MCF-Fas cells from various apoptotic stimuli, such as TNF, Fas, monocytes, and serum starvation. This suggests that BHRF1 is a general inhibitor of apoptosis and functions where the various apoptotic stimuli converge into a common death pathway. To test whether protection against apoptosis is a universal feature of BHRF1, which could contribute to the pathogenesis of EBV, we extended the studies to another cell line, a human

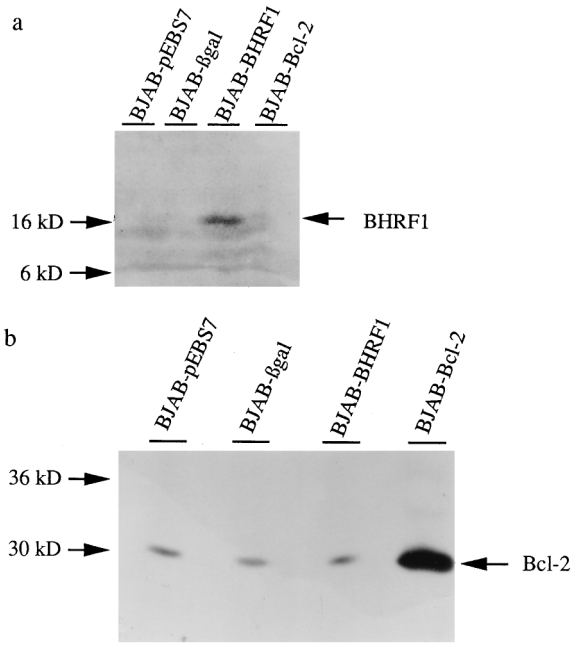

FIG. 6. Detection of BHRF1 (a) or Bcl-2 (b) in transfected BJAB cells by Western blot analysis. BJAB cells were transfected with plasmid pRP-BHRF1 (BJAB-BHRF1), pEBS7-Bcl-2 (BJAB-Bcl-2), pRP-βgal (BJAB-βgal), or pEBS7 (BJAB-pEBS7) by electroporation. Cell lysates from approximately 3×10^6 hygromycin-resistant cells were analyzed by Western blot analysis. Molecular mass markers are indicated on the left.

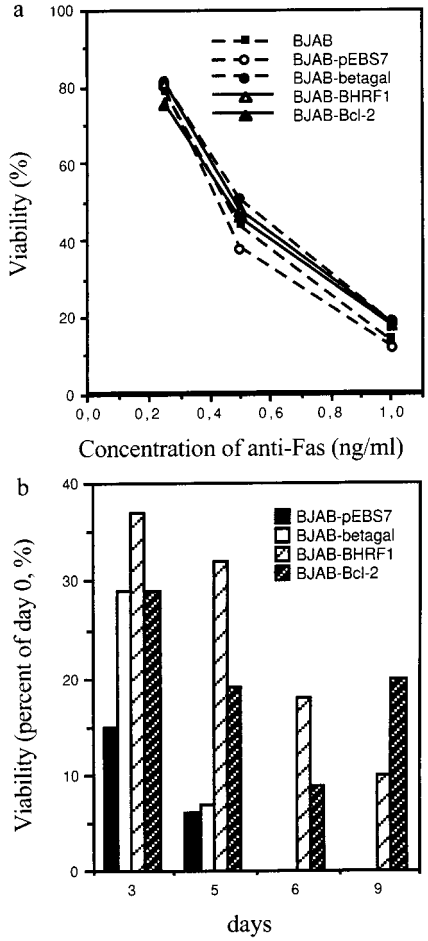

FIG. 7. Expression of BHRF1 or overexpression of Bcl-2 does not protect BJAB cells from anti-Fas-mediated apoptosis (a), but it enhances the survival of serum-starved BJAB cells (b). To measure the sensitivity of cells to anti-Fas, approximately 20,000 cells per well were plated in 96-well plates with the indicated concentrations of anti-Fas antibody with 1 μg of protein A per ml. The percentage of surviving cells was analyzed by MTT assay after a 6-h incubation. In the serum starvation assay, cells were grown in medium containing 0.5% FCS with an initial cell concentration of 400,000 cells/ml. On a daily basis, an aliquot was tested for cell viability. Every second day the MTT assay was accompanied by counting of the cells in trypan blue. Cell pools are as described for Fig. 6.

EBV-negative B-cell line, BJAB, which is sensitive to Fas-mediated cytotoxicity but resistant to killing by TNF.

pRP-BHRF1 was transfected into BJAB cells to create a cell population constitutively expressing BHRF1. In parallel, BJAB cells were transfected with the pRP-βgal expression plasmid or an empty expression vector, pEBS7, creating two control cell populations. In order to compare the effect of BHRF1 with that of Bcl-2, BJAB cells were also transfected with the pEBS7-Bcl-2 expression plasmid. A Western blot analysis showed expression of BHRF1 in BJAB-BHRF1 cells and overexpression of Bcl-2 in BJAB-Bcl-2 cells, compared to the endogenous expression in control transfected cells (Fig. 6). The sensitivity of transfected cells to anti-Fas was tested by a 6-h MTT assay. A comparison between BJAB cells expressing BHRF1 and the parental cell line, the control transfected cells, or Bcl-2-expressing cells did not show any significant difference in sensitivity towards anti-Fas (Fig. 7a). Thus, forced expression of BHRF1 or Bcl-2 did not protect BJAB cells from Fas-mediated killing.

BHRF1 protects BJAB cells from serum starvation. As the expression of BHRF1 did not affect the sensitivity of BJAB cells to anti-Fas-mediated apoptosis, we tested whether BJAB-BHRF1 cells were protected from another apoptotic stimulus, serum starvation. The four BJAB transfectants were then screened for survival over a 9-day period after transfer into medium with a reduced (0.5%) FCS content. An aliquot was tested for cell viability by MTT assay every day from day 1 to day 7. Every second day, the MTT assay was accompanied by counting of the cells in trypan blue to verify that the cells were actually dying by serum starvation. BHRF1-expressing BJAB cells showed significantly enhanced survival compared to control cells (Fig. 7b). After 5 days of incubation, 30% of the BHRF1-expressing cells had survived serum starvation while only 5% of the control cells were alive. After 9 days of incubation, all of the control cells were dead whereas 10% of the BHRF1-expressing cells were alive. The BJAB cells overexpressing Bcl-2 showed a pattern of survival similar to that of BHRF1-transfected cells (Fig. 7b).

DISCUSSION

This study was undertaken to investigate signal transduction pathways involved in apoptosis induced by cross-linking and activation of cell surface receptors for TNF and FasL. Our first aim was to identify gene products that inhibit this pathway. By functional expression cloning, the EBV early-lytic-cycle protein BHRF1 was identified as an antiapoptosis protein. Expression of BHRF1 in MCF-Fas cells led to nearly complete inhibition of cell death induced by TNF, anti-Fas antibody, monocytes, and serum starvation.

EBV is a human herpesvirus with oncogenic potential and is predominantly associated with infection of two target tissues in vivo: B lymphocytes, where the infection is largely nonproductive, and stratified squamous epithelium, in which virus repli-

cation occurs (26). Both cell types are susceptible to EBV-associated transformation, resulting in tumors of B-cell origin, Burkitt's lymphoma and immunoblastic lymphoma, or of epithelial cell origin, nasopharyngeal carcinoma and gastric adenocarcinoma (26).

BHRF1 is an EBV gene product of undetermined function. It is a constituent of all EBV genomes, suggesting that it has an important role in the viral life cycle (21). Spliced BHRF1 transcripts have been identified in latently infected B cells, although the 17-kDa protein has yet to be detected in these cells (35). However, in the early phase of the viral replicative cycle both BHRF1 protein and transcripts are expressed in abundance (35). The expression of BHRF1 is not required for viral replication or B-cell transformation, since recombinant virus in which BHRF1 has been deleted in vitro is both transforming and able to enter the replicative cycle (25). These BHRF1-negative transformed B cells express the full range of viral latent gene products, suggesting an essential role for these proteins (25). The influence of BHRF1, however, is much more likely to be felt in the lytic cycle, in which the protein is abundantly expressed and the expression of latent genes is turned off.

The BHRF1 protein of EBV may be a structural homolog of the Bcl-2 protein. Alignment of BHRF1 and Bcl-2 has revealed some 25% amino acid identity (25), in which the sequences corresponding to the BH1, BH2, and BH3 domains are highly conserved (7).

This and previous studies indicate that BHRF1 is also functionally related to Bcl-2 and the other family member, Bcl-x_L. Previous functional studies have shown that ectopic expression of BHRF1 can block apoptosis induced by various stimuli, such as withdrawal of serum (13), withdrawal of other growth factors, treatment with anticancer agents, infection with heterologous virus (45), and ectopic expression of the tumor suppressor protein p53 (50). This study shows that expression of BHRF1 in MCF-Fas cells nearly completely blocks the apoptotic pathway induced by TNF or anti-Fas even in long-term assays with high concentrations of effector molecules. Moreover, we show that expression of BHRF1 protects MCF-Fas cells from monocyte-mediated killing in a manner similar to that of Bcl-2 and Bcl-x_L. TNF is the major mediator of cell death used by monocytes (1, 19); thus, this result emphasizes the protective effect of BHRF1 against this cytokine. The ability of BHRF1 to protect epithelial cells from TNF- and anti-Fas-mediated apoptosis is supported by a recent publication showing that expression of BHRF1 protected 407 intestinal epithelial cells against apoptosis induced by combination of cycloheximide and TNF or anti-Fas antibody (20). Therefore, BHRF1 may, like Bcl-2 in epithelial cells, be a general inhibitor of apoptosis, acting at a point at which the different signaling pathways converge into a common apoptotic pathway. Surprisingly, however, expression of BHRF1 or overexpression of Bcl-2 did not protect BJAB cells from anti-Fas-mediated apoptosis. BHRF1 and Bcl-2 did, however, increase the survival of BJAB cells deprived of serum. Two other groups have obtained similar results with Bcl-2 in lymphocytes: overexpression of Bcl-2 protected lymphocytes from gamma radiation, staurosporine, and serum deprivation but not from anti-Fas-mediated apoptosis (5, 41). Those authors raised the possibility that Bcl-2 and anti-Fas may regulate distinct pathways to apoptosis. In contrast, however, a recent study shows very clearly that overexpression of Bcl-2 inhibits Fas-induced activation of caspase-3/CPP32 and apoptosis in lymphocytes (2). Our present and previous results (17) showing complete inhibition of anti-Fas-mediated apoptosis by BHRF1, Bcl-2, and Bcl-x_L in MCF-Fas cells suggest that these proteins can regulate Fas-induced apoptosis but that their effect upon it is dependent on the cell type. The different results obtained with MCF-Fas and BJAB cells may be explained through different expression levels of positive and negative regulators of apoptosis. The protective effect seen in the MCF-Fas cells may be due to the high expression levels of BHRF1, which can overcome and prevent homodimerization between preexisting low levels of death-promoting proteins. In contrast, the expression levels of BHRF1 or Bcl-2 in BJAB cells may be too low to prevent homodimerization of a high level of preexisting positive regulators. But why then do we see an effect by BHRF1 and Bcl-2 in serum starvation of BJAB cells? This difference might be explained by the strength of the given stimuli. The anti-Fas stimulus in BJAB cells is very strong, resulting in complete killing of target cells in 6 h, whereas withdrawal of growth factors is a weak death stimulus, with killing taking several days. It is important to note that anti-Fas-induced cell death in MCF-Fas cells takes 24 to 48 h. Thus, one possibility is that the Fas-induced death signal in BJAB cells is so strong that it breaks through the checkpoint regulated by the Bcl-2 family.

Contrary to monocyte-mediated killing, BHRF1, Bcl-2, or Bcl-x_L expression did not protect MCF-Fas cells from LAK-cell-mediated killing. Recent results have demonstrated that the perforin–granzyme B-mediated and Fas-mediated pathways are the two most important mechanisms used by LAK cells in mediating cytotoxicity (39, 53). As anti-Fas killing takes 24 to 48 h, and as BHRF1, Bcl-2, and Bcl-x_L protected MCF-Fas cells from anti-Fas-mediated apoptosis, it is likely that the killing of MCF-Fas cells by LAK cells in the 4-h assay was mediated through the perforin-granzyme B pathway. In the perforin-granzyme B pathway, the LAK cells deliver lethal hits by releasing their granule contents after binding to the target cell. The inability of BHRF1, Bcl-2, or Bcl-x_L to protect MCF-Fas from perforin–granzyme B-mediated cell death may be explained in one of two ways. Pores formed by perforin may induce a loss of cell membrane integrity, which may result in a necrotic form of cell death unpreventable by BHRF1, Bcl-2, or Bcl-x_L, or the entrance of granzyme B to the cell may be responsible for induction of apoptosis and bypass the otherwise protective effect of BHRF1, Bcl-2, or Bcl-x_L. Apoptosis induced by granzyme B cannot be inhibited by Bcl-2, and recent findings suggest that granzyme B-induced cell death occurs via direct activation of downstream caspases, thus bypassing the inhibitory effect of the Bcl-2 family (5, 6). Our data show that BHRF1, Bcl-2, and Bcl-x_L are able to inhibit cytokine-induced activation of caspase-3/CPP32, making the latter hypothesis possible.

Several lines of evidence suggest the involvement of $cPLA_2$ in the cytotoxic pathway of TNF and Fas (11, 17, 32, 52, 58). This idea is further supported by our data showing that expression of BHRF1 inhibited the activation of $cPLA_2$ by TNF and anti-Fas in a manner that paralleled their ability to inhibit cell death. Interestingly, other inhibitors of TNF-induced apoptosis, such as heat shock protein 70, Bcl-2, and Bcl-x_L, also inhibit TNF-induced activation of $cPLA_2$ (16, 17). Activated $cPLA_2$ releases arachidonic acid from the *sn*-2 position of phospholipids, which provides a source for the production of biologically active lipid mediators such as prostaglandins and leukotrienes (12). Thus, BHRF1 may, through its inhibition of $cPLA_2$, also act as an anti-inflammatory factor.

A putative function of BHRF1 could be to protect EBV-infected epithelial cells from TNF- and/or Fas-induced cell death in order to maximize virus production. Both TNF and Fas exhibit antiviral activity and may be two components of the immune system's response to viral infection. Another putative role of BHRF1 may be inhibition of apoptosis during malig-

nant transformation of infected cells. Although BHRF1 (like Bcl-2) alone is insufficient to cause transformation, the surviving cells could provide the substrate for further genetic changes in conventional oncogenes such as c-*myc*. The role of BHRF1 in EBV-infected lymphoid cells remains unclear.

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