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Epstein–Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death

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ABSTRACT **Epstein–Barr virus, a human herpesvirus that persists within the B-lymphoid system, can enhance the survival potential of latently infected B cells *in vitro* through up-regulation of the cellular survival protein Bcl-2. The possibility that an analogous effect is operative in lytically infected cells was suggested by the observation of distant sequence homology between an Epstein–Barr virus-coded early lytic cycle protein, BHRF1, and Bcl-2. Here we show by gene transfer that BHRF1 resembles Bcl-2 both in its subcellular localization and in its capacity to enhance B-cell survival. Thus confocal microscopic analysis of cells acutely cotransfected with BHRF1 and Bcl-2 expression vectors revealed substantial colocalization of the two proteins in the cytoplasm. In subsequent experiments, stable BHRF1 gene transfectants of Burkitt lymphoma cells paralleled Bcl-2 transfectants in their enhanced survival under conditions that induce cell death by apoptosis. Despite their limited sequence conservation, therefore, the two proteins appear to be functionally homologous. We suggest that BHRF1 provides an alternative, Bcl-2-independent, means of enhancing B-cell survival that may operate during the virus lytic cycle.**

Epstein–Barr virus (EBV) persists for life in the immunocompetent host, apparently through the long-term survival of latently infected B cells (1, 2). Since programmed cell death (apoptosis) limits the life span of most B cells *in vivo* (3–5), the virus must either directly access the long-lived memory B-cell pool or promote the entry of infected cells into that pool. The observation that EBV can protect B cells from apoptosis *in vitro* (6) strongly supported this latter possibility. Thus EBV-positive Burkitt lymphoma (BL) cell lines displaying the latency I form of infection, in which only the nuclear antigen EBNA1 is expressed, could readily be induced into apoptosis by signals such as serum withdrawal or treatment with calcium ionophore (5). In contrast BL lines that had progressed on serial passage to a latency III form of infection, with expression of the full spectrum of latent proteins, including nuclear antigens EBNAs 1, 2, 3A, 3B, 3C, and LP and the latent membrane proteins (LMPs) 1 and 2, showed enhanced cell survival under such conditions. Gene transfers into apoptosis-sensitive B-cell lines mapped this protective effect to LMP1 and indicated a likely mechanism, namely, LMP1-mediated up-regulation of the cellular protein Bcl-2 (7). Importantly Bcl-2, a membrane-associated protein with a distinctive pattern of cytoplasmic localization (8, 9), not only enhances cell survival in a variety of experimental situations (7, 9–15) but also appears to be involved in the physiological selection of B cells into memory (16).

Although the above work was concerned only with EBV latent infections, it was interesting to note the existence of an EBV early lytic cycle protein, BHRF1 (17), that itself showed distant sequence homology with Bcl-2 (18). Alignment of the BHRF1 and Bcl-2 sequences revealed some 25% amino acid identity over a 150-aa region of the two proteins (18). The role of the BHRF1 protein in the virus life cycle remains to be determined, however, since recent work with EBV recombinants (19, 20) has indicated that BHRF1 is not required *in vitro* either for virus replication or for virus-induced cell growth transformation to lymphoblastoid cell lines (LCLs). The present work sought to determine whether the distant sequence homology between BHRF1 and Bcl-2 was reflected functionally in the proteins' effects upon cell survival.

MATERIALS AND METHODS

Production of BHRF1 Expression Plasmid. A *Bgl* II–*Dra* I fragment containing the BHRF1 open reading frame (EBV genome coordinates 54,359–55,140) was inserted into the *Eco*RI site in the polylinker of the pSG5 expression vector (21). A BHRF1 expression cassette comprising a simian virus 40 (SV40) promoter, β-globin intron 1, BHRF1 open reading frame, and SV40 polyadenylylation sequences was excised with *Sal* I and then inserted into the unique *Sal* I site of the pHEBO vector (22) to give the BHRF1 expression vector pDH222 (Fig. 1).

Transfections. Transient transfections into COS-1 cells using DEAE-dextran and stable transfections into the EBV-positive BL cell lines Wan-BL (23), Akata-BL (24), and Raji-BL (25) by using electroporation were performed as described (7, 26). For the latter, selection was in hygromycin B at 300 μg/ml (pHEBO-based constructs) or G418 at 2.5 mg/ml (CΔJ-based constructs, ref. 27).

Analysis of BHRF1 and Bcl-2 Expression. *Immunofluorescence.* Transfected COS-1 cells were cultured on microscope slides for 48 hr before air drying and acetone/methanol, 1:1 (vol/vol), fixation at −20°C for 10 min. Slides were incubated for 1 hr at 37°C with a mixture of the Bcl-2-specific IgG1 monoclonal antibodies (mAbs) Bcl-2/100 and Bcl-2/124 (28) and the BHRF1-specific IgG2a mAb 5B11 (17) followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG1 and biotinylated goat anti-mouse IgG2a (Southern Biotechnology Associates), the latter being visualized by Texas red-streptavidin. Fluorescence images were collected and analyzed by scanning confocal microscopy. The MRC-500 (Bio-Rad) system was employed in a dual channel mode and optimized for the fluorescein and Texas red signals based on the 514-nm laser line. To determine areas of colocalization of the fluorescence signals, the Texas red image was modified by the addition of blue color before merging the two images; under these conditions areas of colocalization appeared

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Abbreviations: BL, Burkitt lymphoma; EBV, Epstein–Barr virus; LCL, lymphoblastoid cell line; LMP, latent membrane protein; SV40, simian virus 40; mAb, monoclonal antibody; FCS, fetal calf serum.

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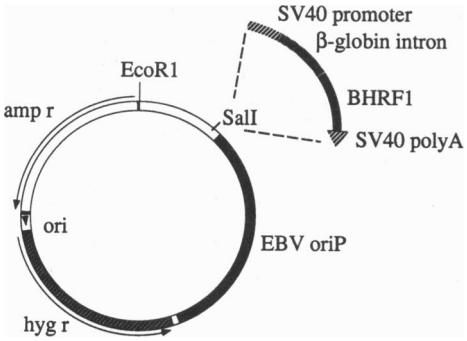

FIG. 1. Diagrammatic representation of the BHRF1 expression vector pDH222. A BHRF1 expression cassette containing the BHRF1 open reading frame downstream of a SV40 promoter and β-globin intron was introduced into the unique *Sal* I site of the pHEBO vector. This vector confers hygromycin resistance (hyg r) and the EBV *ori-p* sequence allows episomal maintenance in EBNA1-positive cells. amp r, Ampicillin resistance.

white, areas of selective Bcl-2 were green, and areas of selective BHRF1 localization were lilac.

Western blot analysis. Detection of Bcl-2 expression using the Bcl-2-specific mAbs Bcl-2-100 and Bcl-2-124 was carried out as described (7).

Immunoprecipitation. For the detection of BHRF1 in stably transfected BL cells, 10^7 cells were incubated for 4.5 hr at 37°C in 3 ml of methionine-free medium supplemented with 2 mM glutamine, 5% (vol/vol) dialyzed fetal calf serum (FCS), and 350 μCi of [^{35}S]methionine (1 Ci = 37 GBq). Cell lysates were immunoprecipitated by incubating with 5B11-coated protein G-Sepharose beads. The immunoprecipitates were separated by Laemmli discontinuous PAGE on a 15% resolving gel and then the gel was fixed, dried, and autoradiographed.

Induction and Detection of Apoptosis. Cells, washed in serum-free medium, were seeded at 3×10^5 per 1-ml well either in control medium (10% FCS), in medium containing suboptimal concentrations of FCS (1% for WAN-BL transfectants or 0.1% for Akata-BL and Raji-BL transfectants) or in 10% FCS medium containing the calcium ionophore ionomycin (Calbiochem) at 1 μg/ml. The percentage of viable vs. apoptotic cells was determined by acridine orange fluorescence (5) from counts of at least 200 cells per individual well, duplicate cultures of each type being sampled at each time point. In all cases, values shown represent the mean with the average deviation between counts always <5%.

RESULTS

Colocalization of BHRF1 and Bcl-2 in Acutely Transfected Cells. Given earlier reports that both BHRF1 (29) and Bcl-2 (8) were membrane-associated, we chose to examine the localization of both proteins when coexpressed to high levels in COS-1 cells. Cells cotransfected with the BHRF1 expression plasmid pDH222 (Fig. 1) and the Bcl-2 expression plasmid pCΔJ Bcl-2 (27) were analyzed 48 hr later by two-color immunofluorescence staining with BHRF1-specific and with Bcl-2-specific mAbs. The two proteins showed substantial, but never complete, overlap in their distribution within perinuclear and cytoplasmic areas. Typical confocal microscopic images from a cotransfected cell are shown in Fig. 2. Fig. 2 *Right* shows image-enhanced profiles of staining intensity for BHRF1 (cell on the right) and Bcl-2 (cell on the left), and Fig. 2 *Left* shows the superimposed immunofluorescence images such that overlapping areas of BHRF1 staining (lilac) and Bcl-2 staining (green) appear white.

Phenotypic Effects of BHRF1 in Stable BL-Cell Transfectants. Given the similar distributions of these distantly related proteins within the cell, we then asked whether BHRF1 might mediate similar effects to Bcl-2 on the cell survival phenotype. EBV-positive BL-cell lines with the latency I form of infection were chosen as target cells for these experiments since (*i*) they can maintain EBV *ori-p*-based plasmids through endogenous expression of nuclear antigen EBNA1 (25), (*ii*) they do not express detectable levels of BHRF1 protein, and (*iii*) they show little or no expression of endogenous Bcl-2 and are readily inducible into apoptosis.

Fig. 3*A Upper* illustrates expression of the 19-kDa BHRF1 protein in two representative clones (BHRF1-8 and BHRF1-11) of the Wan-BL cell line transfected with pDH222. No BHRF1 is detectable in the Wan-BL parental line or in a Bcl-2 transfectant of Wan-BL (Bcl-2-7c), whereas the signal in the positive B95.8 LCL control track is derived from high-level expression of BHRF1 in the 5% subpopulation of cells in lytic cycle. In contrast, expression in the BHRF1 transfectants was homogeneous throughout the cell population when analyzed by immunofluorescence staining, with levels in individual cells being much reduced compared to that seen in the lytic cycle (data not shown). Fig. 3*A Lower* shows a parallel

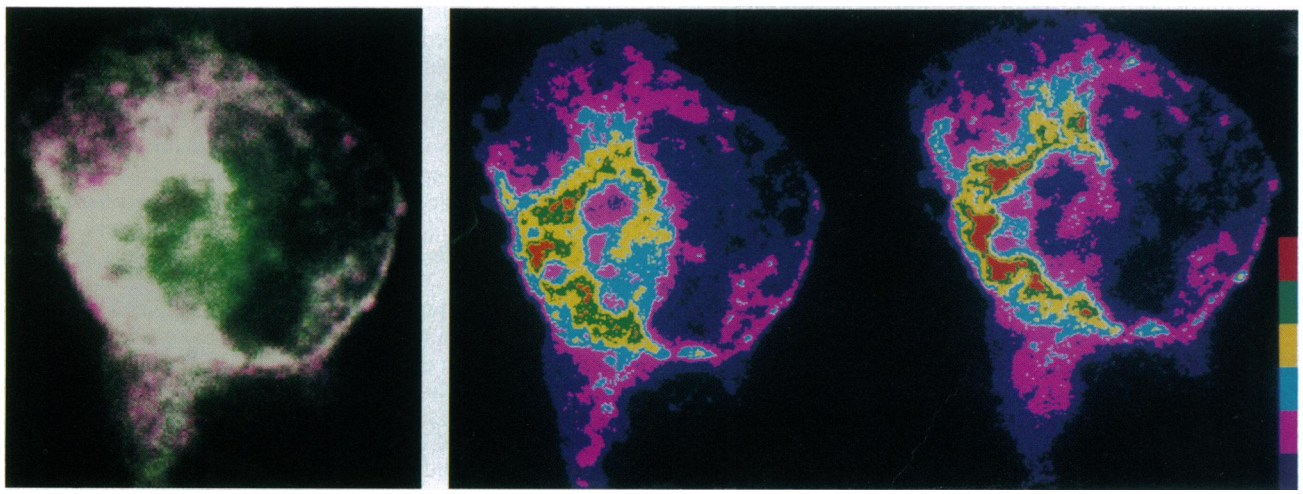

FIG. 2. Comparative localization of BHRF1 and Bcl-2 as visualized by two-color immunofluorescence staining and confocal microscopy of COS-1 cells acutely cotransfected with the BHRF1 expression vector pDH222 and with the Bcl-2 expression vector pCΔJ-Bcl-2. (*Left*) Superimposed images of BHRF1 staining (lilac) and Bcl-2 staining (green) in a representative cell; areas of overlap between the two patterns of staining appear white. (*Right*) Image-enhanced views of the subcellular distribution of Bcl-2 staining (left view) and of BHRF1 staining (right view) in the same cell; color scale of staining intensity (red maximum) is as shown.

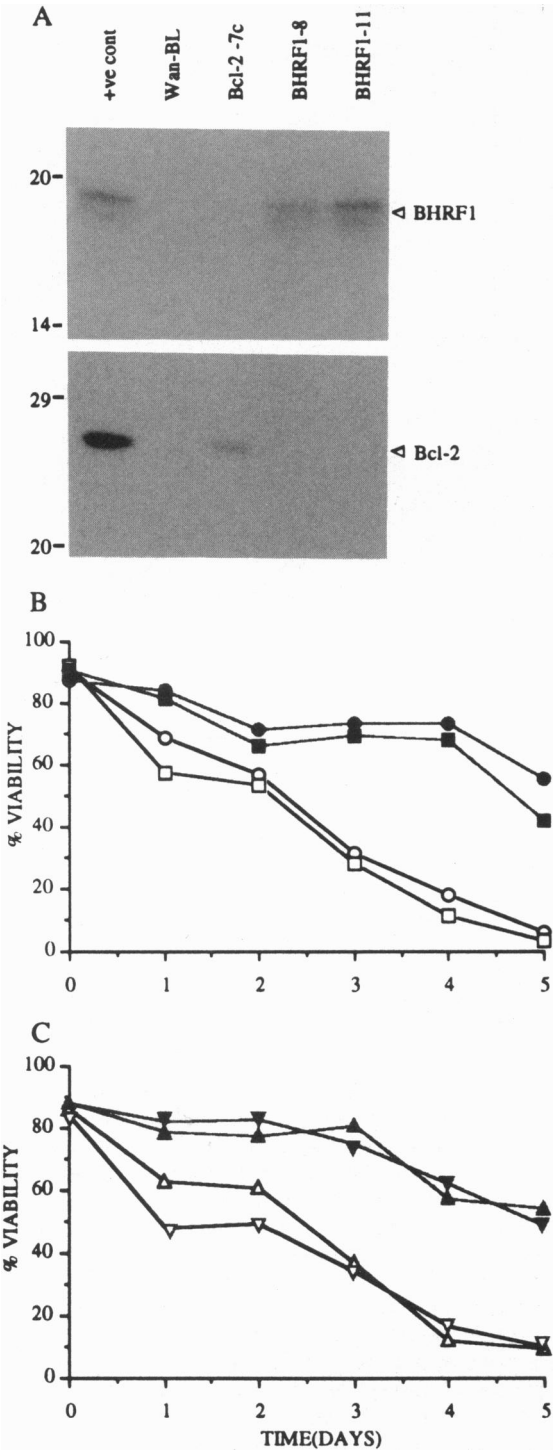

FIG. 3. (*A*) BHRF1 and Bcl-2 expression in Wan-BL transfectants. (*Upper*) Immunoprecipitation of BHRF1 from [^{35}S]methionine-labeled cells using the BHRF1-specific mAb 5B11 and separation by SDS/PAGE on a 15% gel. Note that under such conditions BHRF1 often appears as a doublet (17). (*Lower*) Parallel immunoblot of protein extracts from the same cells separated on a 10% gel and probed with the Bcl-2-specific mAbs Bcl-2-100 and Bcl-2-124. Cell lines analyzed were Wan-BL parental line, Wan-Bcl-2-transfected clone 7c, and Wan-BHRF1-transfected clones 8 and 11. Positive controls were the productively infected LCL B95.8 (*Upper*) and the latently infected LCL X50-7 (*Lower*). (*B*) Cell survival capacity of Wan BHRF1 transfectants after serum depletion. Representative results of cell survival assays for two BHRF1-expressing clones 8 (■) and 11 (●) and for two vector control clones HEBO-2 (□) and HEBO-4 (○) showing cell viability as assessed by acridine orange fluorescence after transfer to medium containing 1% FCS. (*C*) Cell survival capacity of Wan Bcl-2-transfectants after serum depletion. Representative results of parallel cell survival assays for two Bcl-2-expressing clones 7c (▲) and 7a (▼) and for two vector control clones CΔJ-2a (△) and CΔJ-7b (▽).

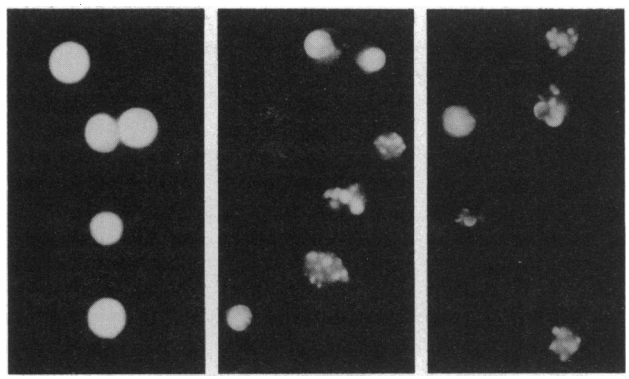

FIG. 4. Induction of apoptosis in BL cells by serum withdrawal and by calcium ionophore. Acridine orange fluorescence of Wan-BL control vector transfectants. (*Left*) Viable cells maintained in 10% FCS. (*Center*) Cells maintained for 4 days in 1% FCS. (*Right*) Cells maintained for 40 hr in 10% FCS in the presence of ionomycin (1 μg/ml).

immunoblot of protein extracts from the same cell populations probed for Bcl-2 expression; this confirmed that the BHRF1 transfectants remained Bcl-2-negative like the parental Wan-BL cell line.

Representative BHRF1-positive and vector control (pHEBO) transfectants of Wan-BL were then screened for survival capacity, measuring viability by acridine orange fluorescence over a 5-day period after transfer into medium with reduced (1%) FCS content. We observed significantly enhanced survival of the BHRF1-expressing clones compared to controls in repeated assays; typical results from one such assay are shown in Fig. 3*B*. For comparison, Fig. 3*C* illustrates the results of assays conducted at the same time on representative Bcl-2-positive and vector control (CΔJSV2) transfectants of Wan-BL established in earlier work (7). The data clearly show that similar enhancement of cell survival can be achieved by BHRF1 as by Bcl-2 when assayed by transfection in the same cell background.

It should be stressed that the 5-day viability counts presented in Fig. 3 do indeed reflect the relative susceptibility of cells to death by apoptosis. Thus virtually all cells dying throughout the assay period in serum-depleted BL cultures were apoptotic by the dual criteria of DNA fragmentation (ref. 7 and data not shown) and acridine orange staining of condensed chromatin. Fig. 4 shows acridine orange staining of a Wan-HEBO control clone in 10% FCS or 4 days after transfer into 1% FCS; continued cell death by apoptosis in the serum-depleted culture is still evident at this time.

In further assays we used the calcium ionophore ionomycin to induce BL-cell death in a more rapid and synchronous manner. Fig. 4 *Right* illustrates the extensive apoptosis seen in a Wan-HEBO control clone after 40 hr in the presence of ionomycin. Enhanced cell survival of Wan-BHRF1 transfectants was again apparent in ionomycin-treated cultures. Representative results of cell viability assays conducted after 20 hr and 40 hr in ionomycin for Wan-BHRF1 vs. Wan-HEBO control clones are shown in Fig. 5. Again the level of protection offered by BHRF1 was similar to that seen in Wan-Bcl-2 transfectants (data not shown).

To examine the generality of BHRF1-mediated effects in BL cells, similar sets of BHRF1-expressing and vector-control transfectants were generated on two further EBV-positive BL-cell backgrounds. These were (*i*) Akata-BL,

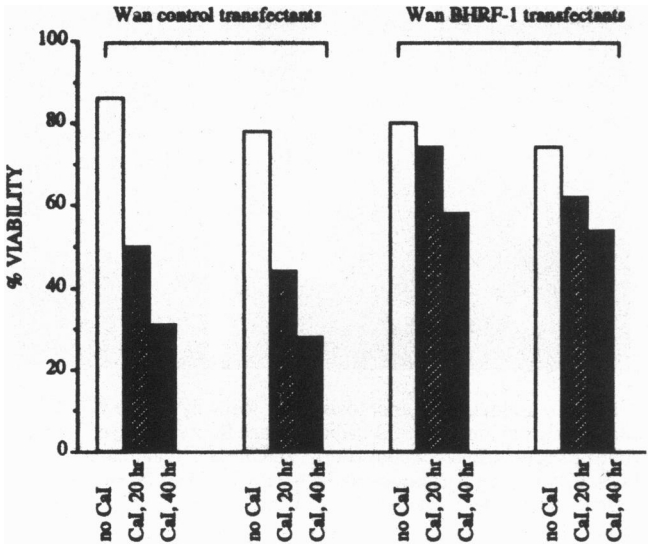

FIG. 5. Cell survival capacity of Wan BHRF1 transfectants after exposure to the calcium ionophore ionomycin. Representative results of cell survival assays for two BHRF1-expressing clones (BHRF1-8 and -11) and two vector control clones (HEBO-2 and -4) as assessed by acridine orange fluorescence. Histograms show cell viabilities in cells cultured in normal medium for 20 hr (□) or in the presence of ionomycin (1 μg/ml) for 20 hr (▨) or 40 hr (■).

which also displays the latency I pattern of EBV infection but naturally expresses trace levels of endogenous Bcl-2 (30) thereby rendering it slightly less sensitive than Wan-BL to apoptosis, and (*ii*) Raji-BL, an unusual line that expresses a wider spectrum of EBV latent proteins including LMP1 but remains susceptible to apoptosis because there has been no concomitant EBV-induced up-regulation of cellular Bcl-2 (S.H., unpublished observations). Fig. 6 presents typical cell survival curves for three BHRF1 transfectants and three control transfectants of Akata-BL (Fig. 6*A*) and of Raji-BL (Fig. 6*B*) after transfer into medium containing 0.1% FCS. Enhanced survival mediated via BHRF1 was evident in multiple clones on both BL-cell backgrounds in repeated serum depletion experiments of this kind and also after treatment with calcium ionophore (data not shown). Once again apoptosis was responsible for the loss of cell viability in these assays. We should also stress that in these and in the earlier experiments with Wan-BL clones, there was no effect of BHRF1 upon the resident pattern of EBV latency or upon the growth rate and viability of cells in standard culture conditions (10% FCS); the BHRF1 phenotype was only observed under suboptimal conditions in which BL cells are naturally induced into apoptosis.

DISCUSSION

There is now considerable interest in the ability of viruses to prolong the life span of the cells they infect (7, 31, 32). EBV persists as a reservoir of latently infected B cells from which reactivation into lytic cycle can provide a source of transmissible infectious virus (1, 2). Given a mechanism whereby EBV may enhance cell survival during latency (7), we became interested in the possibility that an analogous mechanism is operative during lytic cycle. The key observation in this context was the reported sequence homology between the viral early lytic cycle protein BHRF1 and the cellular survival protein Bcl-2 (18). Although this homology is relatively weak with only 25% identity over a 150-aa stretch, here we demonstrate experimentally striking similarities between the two proteins both in their subcellular localization and in their effect upon cell survival.

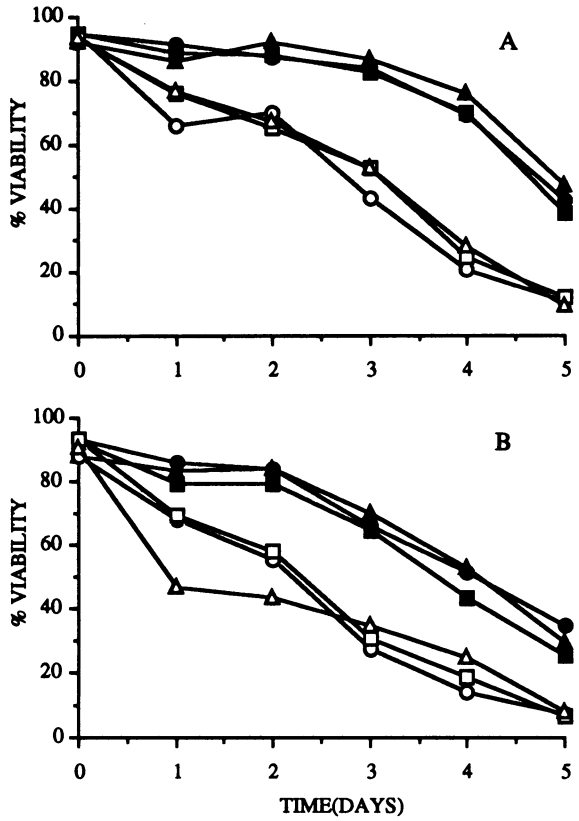

FIG. 6. Cell survival capacity of BHRF1 transfectants of the Akata-BL and Raji-BL cell lines. Representative results of cell survival assays for BHRF1-expressing clones and vector-control clones of Akata-BL (*A*) and Raji-BL (*B*) showing cell viability after transfer to medium containing 0.1% FCS. Individual clones tested were as follows. (*A*) Akata BHRF1 clones 3 (●), 4 (■), and 5 (▲); Akata HEBO control clones 5 (○), 8 (□), and 9 (△). (*B*) Raji BHRF1 clones 2 (●), 4 (■), and 5 (▲); Raji HEBO control clones 2 (○), 8 (□), and 12 (△).

The associations of Bcl-2 with cytoplasmic membranes and with mitochondria are well-established (8, 9) although the functional importance of such associations remains to be determined (33). Current evidence suggests that Bcl-2, which lacks an obvious signal sequence, inserts into membranes via a putative C-terminal transmembrane domain (8). A homologous C-terminal domain can be found in BHRF1 (18) and an earlier biochemical study has suggested that this protein is present in microsomal and mitochondrial subcellular fractions (29). Here we have directly compared the subcellular localizations of the two proteins when coexpressed by gene transfection. Although an association of BHRF1 and Bcl-2 with specific organelles was not formally addressed in the present study, the distribution of immunofluorescence staining for both proteins was consistent with localization to cytoplasmic membranes. Indeed, confocal microscopic analysis showed extensive colocalization of BHRF1 and Bcl-2 in all individual cells in which they were coexpressed (Fig. 2), although we always did observe some areas where staining appeared to be exclusive to one or the other protein.

To examine the possible effects of BHRF1 on cell survival, we expressed the protein in BL-cell lines. Such cells were particularly well suited for this study since they are of human B-lymphoid origin, are inherently sensitive to apoptosis induced by serum withdrawal (5), and furthermore, can have their apoptotic response modulated by Bcl-2 gene transfection (7). Three BL-cell lines were chosen for BHRF1 gene transfection, Wan-BL, Akata-BL, and Raji-BL. Results on all three backgrounds clearly show that BHRF1, like Bcl-2

(7), is capable of enhancing cell survival in culture conditions, such as low serum or addition of ionomycin, which trigger apoptosis (Figs. 3–6). This leads us to conclude that BHRF1 and Bcl-2 are functionally homologous in their effects upon cell survival. Both proteins afforded significant protection in the BL-cell assay, although it is worth noting that their effects were never complete; some loss of viability was still observed in BHRF1 and Bcl-2 transfectants, due to the entry of cells into apoptosis. The protective effects of these proteins in this particular situation may, therefore, be to delay rather than to prevent programmed cell death.

Despite the widespread interest in Bcl-2 as a cell-survival signal, functional domains within the protein and their mechanism of action remain to be identified. The observation of functional homology between BHRF1 and Bcl-2 is, therefore, potentially informative since residues shared by both proteins are likely to contribute to such domains. We further note the recent finding of a survival gene, *ced9*, in the nematode *Caenorhabditis elegans* that functions during development to protect specific cells from programmed cell death (34). The human Bcl-2 not only can replace *ced9* function in the nematode (13) but also shows limited sequence homology with the *ced9* gene (35). It will be interesting to determine the extent of sequence conservation in this emerging family of functionally related genes.

The present findings are also important in the wider context of EBV biology. The BHRF1 gene is present in all natural virus isolates examined to date, yet paradoxically its protein product is not essential *in vitro* either for virus-induced B-cell transformation to LCLs or for full virus replication (19, 20). We propose that BHRF1 could, however, play an important role *in vivo* by prolonging the lifetime of infected cells. Action at the level of latently infected cells appears unlikely since BHRF1 is never expressed in latency I and, despite some transcriptional activity across the open reading frame (17, 36), is not detectable at the protein level in latency III. Furthermore, in this latter situation, any effects of BHRF1 would almost certainly be masked by the activity of the LMP1/Bcl-2 survival pathway. The influence of BHRF1 is, however, much more likely to be felt in lytic cycle where the protein is abundantly expressed as an early antigen; here it may play a role analogous to that of the viral p35 protein expressed during baculovirus replication (31). Thus although productive EBV infection is ultimately cytocidal, the expression of BHRF1 in lytically infected B cells and perhaps also in the virus' other permissive tissue, pharyngeal epithelium (37), may serve to delay cell death and thereby maximize virus production.

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