The Epstein–Barr virus Bcl-2 homolog, BHRF1, blocks apoptosis by binding to a limited amount of Bim

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Current knowledge suggests that the balance between life and death within a cell can be controlled by the stable engagement of Bcl-2-related proapoptotic proteins such as Bak, Bax, and Bim by survival proteins such as Bcl-2. BHRF1 is a prosurvival molecule from Epstein–Barr virus that has a high degree of homology to Bcl-2. To understand how BHRF1 blocks apoptosis, BHRF1 and mutants of BHRF1 were expressed in primary cells and an IL-2-dependent T cell line. BHRF1 bound the Executioner Bak and, when cells were cultured without cytokines, BHRF1 associated with Bim. A point mutation that lost the ability to bind Bak retained its ability to bind Bim and to protect cells. This result demonstrated that it was the capacity of BHRF1 to bind Bim, not Bak, that provided protection. Interestingly, the amount of Bim bound by BHRF1 was minimal when compared with the amount of Bim induced by apoptosis. Thus, BHRF1 does not act by simply absorbing the excess Bim produced while cells prepare for death. Rather, BHRF1 may act either by binding preferentially the most lethal form of Bim or by acting catalytically on Bim to block apoptosis.

death | lymphocyte | Bak

Bcl-2 and its relatives control the life and death of many cell types. The family is divided into 3 groups based on function and the number of Bcl-2 homology (BH) sequences the protein contains. Proteins that inhibit apoptosis are called Protectors and contain BH sequences 1, 2, 3, and 4. Family members that promote apoptosis are known as Messengers and Executioner proteins. Messenger proteins only contain a single homology sequence, BH3, whereas Executioner proteins contain BH1, BH2, and BH3 sequences. Protector proteins, such as Bcl-2 and Bcl-xL, are thought to operate by binding to proapoptotic proteins; thus, preventing their killing activities. The interaction is mediated by a prominent hydrophobic groove formed in part by the BH1 sequence on the surface of Protector proteins that binds to the BH3 sequence of proapoptotic proteins as demonstrated by the interaction between Bcl-xL and the Messenger protein Bim (1). The fate of the cells is thought to be controlled by the relative levels of active Protectors, Executioners, and Messengers (2).

Considering the potent nature of Protectors (3–5), it is not surprising that viruses have evolved homologs of Bcl-2 family members (6, 7). For example, Epstein–Barr virus (EBV) encodes the Bcl-2 related protein, BHRF1, a protein that appears to prevent apoptosis of cells during initial stages of infection (8) and after exposure to many apoptosis-inducing events (9–11). Previous mutational studies showed that mutations in the BH1 sequence of BHRF1, a sequence that is part of the potential BH3-binding site of BHRF1, blocked its protective capacity (12). Likewise, a recent study has demonstrated an interaction between BHRF1 and the BH3-only protein Bim and Puma (13). These experiments suggest that BHRF1 might bind BH3-only proapoptotic proteins via its potential BH3 binding groove, just as Bcl-2 and Bcl-xL do. However, the effects of the BHRF1 mutations with respect to cellular protein interactions were not established in the former article (12), and mutational studies were not performed in the latter article (13). Thus, we cannot

conclude unequivocally from these published studies that Bim binds to the BH3 binding groove of BHRF1, or that BHRF1 protects cells from death by interfering with the actions of Bim.

In an attempt to resolve these contradictions, the functions of BHRF1 and BHRF1 with mutations in its BH3-binding groove were studied in lymphocytes. Here, we show that BHRF1 protects both B and T cells from death. In a lymphocyte cell line, BHRF1 binds the Executioner Bak and, when the cell receives signals to die, the Messenger Bim. Mutational studies show that both these interactions involve the conventional, BH3-binding groove of BHRF1. However, the mutants do not have the same hierarchy of effects on Bak and Bim binding. The relative protective potencies of BHRF1 and BH1 sequence mutants of BHRF1 correlate better with the ability of the molecules to bind Bim rather than their ability to bind Bak. Thus, BHRF1 appears to protect lymphocytes from death by binding Bim, not Bak. Remarkably, BHRF1 manages this task even though it binds only a small amount of the Bim induced by apoptotic stimuli in the cell, suggesting that BHRF1 may protect cells by binding to some crucial, lethal fraction of all of the Bim in the cell, rather than the general pool of Bim.

Results

BHRF1 Protects Primary T and B Cells from Apoptosis. To study the protective effects of BHRF1, retrogenic chimeric mice were generated by transducing bone marrow with MiT-based retroviruses (14) expressing BHRF1 or Bcl-2. These viruses allow the tracking of transduced cells by concomitant expression of the transgene and the cell surface protein Thy1.1. All subsequent transductions use the MiT vector unless stated otherwise.

Cultured T cells die by apoptosis unless supplemented with cytokines like IL-7 (15–17). To test whether BHRF1 could protect primary T cells from culture without cytokines, T cells from retrogenic chimeras were cultured with or without IL-7, and viability was assessed over time. T cells expressing the empty vector died unless cultured with IL-7 (15, 16) (Fig. 1A), whereas expression of Bcl-2 or BHRF1 was protective (Fig. 1A). Because T cell death in culture depends on Bim (18, 19), we hypothesized that BHRF1 might block the actions of Bim, and consequently, block other forms of Bim-dependent apoptosis.

The death of activated T cells requires Bim (20). To test whether BHRF1 could protect activated T cells, V β 8 transgenic mice (V β DO mice) were injected i.v. with 100 μ g of staphylococcal enterotoxin B (SEB). One day later, T cells were isolated from spleen and lymph node, and transduced with retroviruses encoding Bcl-2, BHRF1, or the empty vector (Thy1.1 only). V β 8

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Fig. 1. BHRF1 protects primary T cells from cytokine deprivation and activated T cell autonomous death. (A) Naïve T cells from mice chimeric for bone marrow cells transduced with bicistronic retroviruses expressing Thy1.1 and BHRF1, Thy1.1 and Bcl-2, or the empty vector and Thy1.1 were isolated from spleen and lymph node. Cells were cultured for the indicated time, and survival was determined by the forward and side-scatter properties of the cells (36). The percentages of surviving CD19⁺ Thy 1.1⁺, CD3⁺ CD4⁺, or CD3⁺ CD4⁺ (CD8 cells) cells are indicated. (B) Spleen and lymph node T cells from Vβ8.2 transgenic mice (VpDO) were isolated 24 h after i.v. injection of 100 μg of SEB. Cells were transduced with retroviruses as above, cultured for 48 h, and their survival determined as above. The percentages of surviving cells expressing TCR Vβ8, Thy 1.1 and CD4 or CD8 are indicated. Results shown are the means and SEM of 3 independent experiments. * *P* < 0.05.

T cells expressed approximately equivalent levels of Thy1.1 after transduction, indicating similar levels of ectopic gene expression (see supporting information [Fig. S14](#)). T cells were cultured for an additional 48 h, and transduced cells were assessed for survival. Vector only expressing cells died, whereas cells transduced with Bcl-2 or BHRF1 were protected ([Fig. 1B](#)). Activated T cells expressing BHRF1 from retrogenic bone marrow chimeric mice were also resistant to SEB-induced death ([Fig. S2](#)).

BHRF1 Protects Cells from Bim-Dependent Apoptosis via Its BH3 Binding Groove

Binding Groove. Protective Bcl-2 family members are believed to act by binding, via a hydrophobic groove, the BH3 regions of proapoptotic proteins. The base of the groove of the protector is formed by its BH1 sequence. Shown in Fig. 2A is the alignment of the BH1 sequences of Bcl-xL and BHRF1. Structural studies have suggested that the hydrophobic groove of BHRF1 is inaccessible; however, mutational analysis has indicated the region is important for its function (12, 21, 22). To test whether the BH1 region of BHRF1 is required for protection, BHRF1 was mutated at residues analogous to those responsible for hydrophilic interactions between Bcl-xL and Bim (1), specifically, L98, G99, and R100. In total, 4 alanine substitution mutants were made: 3XA (mutations at L98, G99, and R100), L98A, G99A, and R100A. (Fig. 2A).

Each mutant was tested for efficacy in several ways. To test effects on B cells, bone marrow cells from MD4 mice that express a transgenic B cell receptor for hen egg lysozyme (HEL) were cultured in IL-7 to produce HEL-reactive immature B cells. Cells were transduced with retroviruses expressing Bcl-2, BHRF1 or BHRF1 BH1 mutants, or the empty vector (Thy1.1 only).

Fig. 2. BHRF1 protects bone marrow B cells from deletion via its BH1 sequence. (A) The BH1 sequences of Bcl-xl and BHRF1 are aligned. In magenta are the residues of the BH1 sequence, L98, G99, and R100, that were mutated to alanine. A mutant expressing all 3 substitutions, 3XA, was also generated. (B) By using retroviral transduction, wild-type and BH1 sequence mutants of BHRF1 were expressed in bone marrow cells from MD4 transgenic mice cultured in IL-7. After 5 days of culture, B cells were either stimulated or not with 1.0 μ g of HEL; 48 h later, survival of cells was determined by their forward and side-scatter properties. Stimulated cultures were analyzed in triplicate, whereas only 1 resting culture was analyzed. The percentages of surviving Thyl.1⁺, CD43⁻, CD19⁺, IgM^{low} cells are shown. Error bars represent the SEM of 3 independent experiments. Vector and G99A cultures were tested in 2 independent experiments. *, $P < 0.05$.

Transduced cells expressed approximately equal levels of Thyl.1, suggesting similar expression of introduced genes (Fig. S1). After 7 days of culture, without stimulation, cells expressing Bcl-2, BHRF1, and the L98A and R100A mutants were almost completely protected from cell death (Fig. 2B). However, cells expressing the vector alone, or the BHRF1 3XA or G99A mutants had begun to die. As expected (17), cell death was increased when surface Ig on the cells expressing the empty vector was engaged by HEL. Death also increased in cells expressing 3XA or G99A, whereas cells expressing Bcl-2, BHRF1, L98A, or R100A were protected. Although it remains unclear what is killing B cells cultured without antigen, apoptosis of immature B cells by BCR cross-linking is mediated by Bim (17). Thus, combined with the experiments on T cells, this experiment suggested that BHRF1 protects lymphocytes from death via inhibition of Bim, probably by binding Bim via its BH3-binding groove.

The IL-2-Dependent Cell Line, HT-2, Dies via Bim in the Absence of IL-2.

To have enough cells to perform biochemical analyses of the interactions between BHRF1 and cellular proteins, the IL-2-dependent mouse T cell line, HT-2, was used (23, 24). To evaluate the importance of Bim in the death of HT-2 cells, cells were transduced with retroviruses coding for GFP alone, or GFP plus Bim shRNA. GFP+ cells were sorted and lysed in CHAPS. Lysates were western blotted for Bim and β -actin. Fig. 3A shows a clear reduction of Bim protein in cells expressing the Bim shRNA. To test whether reduced Bim levels protected cells, HT-2 cells expressing Bim shRNA or the empty vector were cultured with or without IL-2 for 18 h. HT-2 cells expressing Bim shRNA were protected from cytokine withdrawal-induced apoptosis (Fig. 3B).

Fig. 3. Death of HT-2 cells after IL-2 withdrawal depends on Bim. (A) HT-2 cells were transduced with LMP-based retroviruses (Open Biosystems) expressing only GFP or GFP and Bim shRNA, and sorted for GFP. Bim and actin protein levels in the vector or Bim shRNA expressing HT-2 cells were measured by Western blotting. (B) Sorted, GFP or Bim shRNA expressing HT-2 cells were cultured with or without IL-2 for 18 h, and assessed for survival by level of 7AAD incorporation. Shown are the means and SEM of 3 identically treated sets of cultures in 1 representative experiment of 2. *, $P < 0.05$.

BHRF1 Protects HT-2 Cells from Death by Binding Bim. To verify that BHRF1 used its BH3 binding groove to protect HT-2 cells from death, HT-2 cells were transduced with retroviruses expressing a FLAG-BHRF1 or FLAG-BH1 mutants of BHRF1. Thy1.1+ transduced cells were isolated by sorting or cloning by limiting dilution. Thy1.1 levels varied slightly between cell lines (Fig. S1C), but BHRF1 and its mutants were expressed at similar levels of protein (Fig. S3A). Cells were cultured in the absence of IL-2, and assessed for death over time by measurement of mitochondrial membrane potential ($\Delta\Psi$), and 7AAD incorporation. When cultured without IL-2, cells expressing the empty vector or the 3XA mutant died within 18 h. Cells expressing G99A were partially protected, and cells expressing R100A and

L98A were fully protected at this time. Only cells expressing BHRF1 were alive at 30 h (Fig. 4A). Thus, protective capacity of BHRF1 in HT-2 cells depends on its BH1 sequence; therefore, BHRF1 probably protects cells by interacting with proapoptotic Bcl-2 family members.

To find out whether BHRF1 binds such proteins, FLAG-BHRF1 and its FLAG-BH1 mutants were immunoprecipitated. Western blots showed that FLAG-BHRF1 and its mutants were precipitated at approximately equal levels (Fig. S3A and Fig. S4), and that, in cells cultured with IL-2, BHRF1 interacted with the executioner protein, Bak, but not Bax (Fig. S3 and Fig. S5). The 3XA and G99A and R100A mutants bound no appreciable amount of Bak (Fig. S3). Similar results, albeit with less Bak bound by BHRF1 mutants, were seen in cells cultured without IL-2. Bak binding by the 3XA mutant after culture without IL-2 could not be evaluated because all of the cells were dead. The R100A result was particularly striking, because this mutant protected HT-2 cells from death in the absence of IL-2 quite well (Fig. S3 and Fig. 4A). This contradiction, illustrated diagrammatically in Fig. S3E, suggests that that BHRF1 does not protect cells from death, due to cytokine withdrawal, by binding Bak, an idea that is supported by the fact that BHRF1 protects Bak^{-/-} T cells from death (Fig. S6).

BHRF1 also bound to Bim, and the amount of Bim bound by BHRF1 proved to be the important correlate of protection. In HT-2 cells cultured with IL-2, BHRF1 bound <1% of Bim within the cells (Fig. S7). However, in the absence of IL-2, wild-type BHRF1 increased binding to Bim_{EL}, the phosphorylated form of Bim_{EL} (Bim_{ELP}) and Bim_L (Fig. 4B and C). This result was confirmed by immunoprecipitation of Bim from cells expressing FLAG-BHRF1 and Western blotting for FLAG (Fig. S8). In HT-2 cells cultured without IL-2, all mutants of BHRF1 bound significantly less Bim_{ELP} and Bim_L than wild-type BHRF1 did ($P < 0.05$; see Fig. 4B and C). A similar trend was observed for

Fig. 4. BHRF1 protects against cytokine deprivation induced apoptosis by binding Bim. (A) HT-2 cells were transduced with the retroviruses described in Fig. 2, and enriched for Thy 1.1 by magnetic bead selection (3XA, L98A, G99A, and R100A) or cloned by limiting dilution (wild-type BHRF1, vector). Cells were cultured without IL-2, and tested for their mitochondrial membrane potential by staining with mitotracker CMX ros or DiOC₆ and 7AAD. Depicted are the percentages of cells that were 7AAD negative, with intact mitochondrial membrane potential. Shown are the means and SEM of results from 3 to 5 independent experiments. †, $P < 0.01$ between sample and vector. *, $P < 0.01$ between BH1 mutant and wild-type BHRF1. (B) HT-2 cells expressing FLAG-BHRF1 and FLAG-BHRF1 BH1 sequence mutants were cultured without IL-2 for 20 h. Live cells were enriched and lysed in 2% CHAPS buffer. Lysates were immunoprecipitated with anti-FLAG beads. Shown are the blots of immunoprecipitates (IP), supernatants from the immunoprecipitations (FT), and the 2% CHAPS lysates before manipulation (wcl). Membranes were western blotted for Bim and FLAG. A representative of 3 independent experiments is shown. Bim_{ELP}, Bim_{EL}, Bim_L, and Bim_S isoforms are indicated. *, $P < 0.01$ between BH1 mutant and wild-type BHRF1. (C) Western blottings were quantified by using a laser scanning imaging system. The percentage of the total amount of Bim isoforms/cell precipitated by BHRF1 and its BH1 sequence mutants were calculated by finding the ratio between bound proteins and the total amount of protein, extrapolated from the sum of the flow through (FT) and the amount precipitated (IP). Shown are the means and SEM of 3 independent experiments. (D) Plotted is the percentage of the total amount of Bim_{ELP} bound by BHRF1 and its BH1 sequence mutants with respect to the percentage of surviving cells after 30 h without IL-2. The arrowhead indicates the R100A mutation that, despite a loss of Bak binding, retains its protective function and binds Bim. †, a contaminating band from the light chain of the FLAG precipitating antibody. This signal was subtracted from Bim_{EL} signals during quantification. P values were obtained by 2-way, unpaired student's t test between indicated BHRF1 mutant and wild-type BHRF1. *, $P < 0.05$.

Fig. 5. Culture of HT-2 cells without IL-2 increases the amount of Bim_{EL} per cell, but does not affect the amount of Bak per cell. (A) HT-2 cells expressing BHRF1 or just the vector were cultured with or without IL-2 for 20 h. Cells were solubilized in 2% CHAPS and blotted for Bim, Bak, and Beta-actin by using HRP secondary antibodies, whereas Bcl-2 was detected with fluorophore conjugated antibodies. (B) Western blottings were quantified by using a CCD imaging system, and the amounts of the indicated protein were normalized to actin levels. Results shown are the means and SEM of 3 independent experiments, each run on a single blot. *P* values were obtained by 2-way, unpaired student's *t* test comparing indicated protein levels in cells expressing BHRF1 cultured with or without IL-2 for 20 h. *, *P* < 0.01.

Bim_{EL}. The R100A mutant bound Bim to a degree that correlated with its protective effects. When the amount of Bim_{EL} bound by BHRF1 or its mutants was plotted relative to the ability of the individual molecule to protect, a strong correlation between protection and Bim binding was observed (Fig. 4D). This result indicates that BHRF1 protects cells by binding to Bim.

BHRF1 Does Not Bind All of the Bim Induced in Cells that Are Destined to Die, Yet Prevents Cell Death. HT-2 cells expressing BHRF1 or the empty vector were cultured with or without IL-2 for 20 h. CHAPS soluble lysates were blotted for Bak, Bim, Bcl-2, and actin. As shown in Fig. 5A and quantified in Fig. 5B, the levels of all forms of Bim, except Bim_L, increased 2- to 3-fold, whereas the Bcl-2 amounts per cell (relative to actin) fell, in the absence of IL-2. These phenomena have been described previously by others and ourselves (20, 25, 26). Levels of other Bcl-2 like proteins, such as the executioner protein Bak, were unchanged (Fig. 5).

Thus, the fact that BHRF1 binds more Bim in HT-2 cells lacking IL-2 may be due to the increased amounts of Bim and/or the drop in Bcl-2 and/or the creation of some special form of Bim that binds preferentially to BHRF1 in the former cells. The idea that Bcl-2 and BHRF1 may compete for binding to Bim is supported by the fact that BHRF1 binds quite a lot of Bim in the Bcl-2 negative human B cell line Daudi (27), even if the cells are healthy (Fig. S9A and B). To test the idea more directly, Bcl-2 was overexpressed by transduction in HT-2 cells expressing BHRF1. The overexpression of Bcl-2 greatly reduced, but did not abrogate, Bim binding to BHRF1 after withdrawal of IL-2 (Fig. S9C).

We calculated whether the increase in Bim bound by BHRF1 correlated with the increase in Bim protein caused by the absence of IL-2. BHRF1 binds <1% of total Bim_{EL} (including the Bim_E and Bim_{EL} forms) in cells cultured with IL-2 (Fig. 6, Fig. S7). After 20 h of culture without IL-2, BHRF1 bound 14% of the total Bim_{EL} (Fig. 6). However, the amount of Bim_{EL}/cell

Fig. 6. BHRF1 binds a limited amount of the Bim induced in apoptotic cells. Data generated from experiments described in Fig. 4 are plotted. HT-2 cells were withdrawn or not from IL-2 for 20 h. The amount of Bim_{EL} bound by BHRF1 is inset within the amount of total Bim_{EL} in the cells. Shown are the means and SEM generated from 3 independent experiments for percentages bound as described in Fig. 4.

had increased 4-fold (Fig. 5), probably because of an increase in the amount of Bim_{EL} in the cell and modification of Bim_{EL} to the more lethal nonphosphorylated form, Bim_{EL} (28). Thus, although BHRF1 increased its binding to Bim, the vast majority of extra Bim produced during apoptosis was not bound to BHRF1. This result was not due to saturation of BHRF1, because immunoprecipitation of Bim revealed that only a small amount of BHRF1 was interacting with Bim whether or not the cells were cultured with IL-2 (Fig. S8). Thus, despite the limited interaction between BHRF1 and Bim, BHRF1 protects the cells.

Discussion

BHRF1 appears to prevent death by binding Bim. Although BHRF1 binds 2 different proapoptotic proteins, Bim and Bak, the ability of BHRF1 mutants to prevent death due to absence of survival cytokines correlates with their patterns of binding to Bim, not Bak. However, remarkably, BHRF1 protects even though it binds only a small proportion of the Bim within the cell. Current dogma posits that apoptosis proceeds when the amount of proapoptotic Bcl-2 molecules surpasses the absorptive capacity of prosurvival Bcl-2 molecules. This balance is shifted after induction of apoptosis; in HT-2 cells, Bcl-2 levels fall and there is a substantial increase in Bim. If BHRF1 operated according to the current rationale, BHRF1 would need to bind the excess of Bim produced; thus, restoring the balance. Instead, BHRF1 binds only a small amount of Bim.

How can BHRF1 protect cells from death when it binds such a small fraction of the Bim? Perhaps BHRF1 prevents cell death by binding to some other protein, and that the results observed here for Bim, although strongly suggestive that Bim is the crucial interactive partner, actually reflect the relative interactions of BHRF1 and its mutants with something else. However, this other protein cannot be one of the other usual suspects, because, as cells initiate apoptosis, BHRF1 molecules interact slightly less with Bak, and at no stage does BHRF1 bind Bax (Fig. S5).

Culture without IL-2 dramatically increases the amount of Bim in the cells. Most of the extra Bim may be bound by other antiapoptotic proteins, and BHRF1 may protect cells from death by binding the small amount of free Bim that is not dealt with by the normal prolife endogenous proteins. We think this explanation is an unlikely possibility, because Bcl-2 does not bind more Bim in the IL-2 deprived cells, and none of the other prolife candidates tested go up in amount/cell when IL-2 is absent. Therefore, in order for this explanation to be correct, the cell must contain a very large, and, in the IL-2 incubated, unused reservoir of proteins that serve to absorb the extra Bim when IL-2 is withdrawn.

We propose that BHRF1 protects cells by binding a particularly lethal portion of the Bim in the cell. Bcl-2 must also bind this target, because overexpression of Bcl-2 also protects at least primary lymphocytes from death (Figs. 1 and 2). This lethal form must be just a small proportion of the Bim in the cells, because BHRF1 protects by binding so little Bim. How might this lethal portion be distinct from the rest of the Bim? A recent report has described a noncanonical interaction between a fragment of Bim and Bax (29), in which Bim did not bind the BH3 binding groove of Bax, but, instead, at a region on the opposite side of Bax. Perhaps Bim in the conformation that binds in this way to Bax is the target of BHRF1. In support of this idea is the fact that BHRF1 does not bind Bim until apoptosis begins. This result suggests that some change in the status of Bim is induced in cells cultured in the absence of cytokines, and this change makes the protein more likely to be engaged by BHRF1.

Alternatively, BHRF1 may act in a catalytic fashion by promoting inactivation of Bim in such a way that the Bim continues to be inactive after it has been released by BHRF1. This mode of action would be analogous to the supposed ability of denatured prion proteins to denature other precursor prions. In support of this idea, changes in conformation occur frequently in Bcl-2 family members and affect their interactions. For example, Bim, Bak, and Bax have been shown to undergo radical structural changes on dimerization (1, 30). Thus, protection may be mediated through BHRF1 promoting a permanent change in conformation of Bim to a shape that cannot participate in cell death.

Although BHRF1 binds the proapoptotic protein, Bak, we show here that this interaction is not involved in the protective effects of BHRF1 in cytokine deprived cells. This observation is not surprising, because Bax can substitute for Bak in this kind of lymphocyte death (31, 32), and BHRF1 does not bind Bax. Why, then, does BHRF1 interact with an apparently irrelevant protein? Most likely, the interaction is relevant, but not during apoptosis induced by cytokine withdrawal, but rather during some other potentially apoptotic threat to EBV-infected cells. The nature of this other form of death remains to be determined. In this context, it is interesting to note that, perhaps not coincidentally, another viral antiapoptotic protein from vaccinia, F1L, also binds both Bak and Bim, and the protective capacity of F1L remains in the absence of Bak (34, 35).

Our findings also suggest considerable plasticity of the structure of BHRF1. Clearly, the groove previously thought to be rendered inactive by steric hindrance (21) is involved in binding to Bim and Bak. Data of others support this idea, because the groove of BHRF1 that is closed in the NMR structure (21) is open in the solved structure of BHRF1 bound to the BH3 region of Bim (PDB ID code 2V6Q).

In summary, BHRF1 appears to protect cells in a unique fashion. BHRF1 constitutively associates with Bak, but this interaction is irrelevant for protection from apoptosis induced by cytokine withdrawal. Instead, BHRF1 interacts with its target, Bim, only when apoptosis is initiated. This result suggests that

apoptosis induces a distinct subfraction of Bim, and it is this altered state of Bim that is targeted by BHRF1.

Materials and Methods

Bone Marrow Chimera Generation and in Vitro Transduction of Activated T Cells.

Mice were purchased from The Jackson Laboratory or bred in our facility under pathogen free conditions approved by the National Jewish Animal Care and Use Committee. C57BL/6 mice were treated i.p. with 5-FU at 0.15 mg/g 5 days before sacrifice, and harvest of their bone marrow. IL-6, stem cell factor and IL-3 were added to cell cultures. Retroviruses were made in Phoenix cells, and cells were transduced twice as previously described (14, 20). Syngeneic hosts were treated with 950 rads, and then received (i.v.) 2.5×10^5 stem cells and 1×10^6 Rag $1^{-/-}$ bone marrow cells. Mice were killed at 6 to 12 weeks.

For SEB activation experiments, V β DO transgenic mice were injected i.v. with 100 μ g of SEB in 200 μ L of BSS; 1 day later, spleens and lymph nodes were harvested, and T cells were purified by nylon wool and transduced. Cells were analyzed 48 h later.

Cell Culture and Enrichment. In survival experiments with cells from chimeric mice, spleen and lymph node cells were depleted of red blood cells. In some experiments, T cells were enriched by magnetic bead selection of CD43⁺ cells (Miltenyi). In B cell stimulation experiments, bone marrow from MD4 mice was cultured overnight in IL-7, removed from stroma, transduced as above, and maintained in IL-7; 4 days later, 1 μ g/mL HEL was added or not. The cells were analyzed 24 and 48 h later. HT-2 cells were transduced as above and enriched for Thy 1.1 by staining with Thy1.1 PE, and anti-PE magnetic bead selection (Miltenyi), or single cell cloning via limiting dilution. The cells were maintained in complete media supplemented with IL-2.

Western Blotting and Immunoprecipitation. Cells were lysed at room temperature for 15 min in 2% CHAPS/PBS with protease inhibitors: 0.1 mM PMSF and complete mini (Roche). Lysates were centrifuged at $10,000 \times g$ for 10 min and immunoprecipitated by means of the FLAG epitope fused to the N terminus of Bcl-2, BHRF1, or its variants by using 40 μ L of wet M2 beads (Sigma). After 2 h, beads were washed 3 times with 200 μ L of cold lysis buffer. Proteins were eluted by using SDS loading buffer without denaturing agents. PAGE was performed, and then proteins were transferred to PVDF. Membranes were blotted and quantified as described in *SI Materials and Methods*.

Cell Staining for Flow Cytometric Analysis. HT-2 T cell lines were incubated with antibodies and 7AAD at 0.33 mg/mL for 15 min at 4 °C. In some cases, 25 nM DiOC₆ (Calbiochem) or 50 μ M Mitotracker CMX ROS (Invitrogen) was added to cultures for 15 min at 37 °C. Cells stained with Mitotracker were chased with fresh media for 15 min at 37 °C to prevent nonspecific staining. Antibodies used are listed in *SI Materials and Methods*.

Bim Short Hairpin RNA. The Bim shRNA retroviral vector was a kind gift from Drs. Paul Waterman and Yosef Refa'eli. The following sequence was cloned into the LMP retroviral vector (Open Biosystems): Sense oligo, TCGAGAAGG-TATATTGCTGTGACAGTGAGCGGGAGGGTGTTC AAATGATAGTGAAGC-CACAGATGTATCATTTGCAACACCTCTGCTACTGCTCGG.

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1. Liu X, Dai S, Zhu Y, Marrack P, Kappler JW (2003) The structure of a Bcl-xL/Bim fragment complex: Implications for Bim function. *Immunity* 19:341–352.
2. Adams JM, Cory S (1998) The Bcl-2 protein family: Arbiters of cell survival. *Science* 281:1322–1326.
3. Nunez G, et al. (1989) Growth- and tumor-promoting effects of deregulated BCL2 in human B-lymphoblastoid cells. *Proc Natl Acad Sci USA* 86:4589–4593.
4. Nunez G, et al. (1990) Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J Immunol* 144:3602–3610.
5. Vaux DL, Cory S, Adams JM (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335:440–442.
6. Polster BM, Pevsner J, Hardwick JM (2004) Viral Bcl-2 homologs and their role in virus replication and associated diseases. *Biochim Biophys Acta* 1644:211–227.
7. Cuconati A, White E (2002) Viral homologs of BCL-2: Role of apoptosis in the regulation of virus infection. *Genes Dev* 16:2465–2478.

8. Altmann M, Hammerschmidt W (2005) Epstein-Barr virus provides a new paradigm: A requirement for the immediate inhibition of apoptosis. *PLoS Biol* 3:e404.
9. Tarodi B, Subramanian T, Chinnadurai G (1994) Epstein-Barr virus BHRF1 protein protects against cell death induced by DNA-damaging agents and heterologous viral infection. *Virology* 201:404–407.
10. Henderson S, et al. (1993) Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. *Proc Natl Acad Sci USA* 90:8479–8483.
11. Foghsgaard L, Jaattela M (1997) The ability of BHRF1 to inhibit apoptosis is dependent on stimulus and cell type. *J Virol* 71:7509–7517.
12. Khanim F, et al. (1997) BHRF1, a viral homologue of the Bcl-2 oncogene, is conserved at both the sequence and functional level in different Epstein-Barr virus isolates. *J Gen Virol* 78:2987–2999.
13. Flanagan AM, Letai A (2008) BH3 domains define selective inhibitory interactions with BHRF-1 and KSHV BCL-2. *Cell Death Differ* 15:580–588.

14. Mitchell TC, et al. (2001) Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat Immunol* 2:397–402.
15. Boursalian TE, Bottomly K (1999) Survival of naive CD4 T cells: Roles of restricting versus selecting MHC class II and cytokine milieu. *J Immunol* 162:3795–3801.
16. Vella AT, Dow S, Potter TA, Kappler J, Marrack P (1998) Cytokine-induced survival of activated T cells in vitro and in vivo. *Proc Natl Acad Sci USA* 95:3810–3815.
17. Enders A, et al. (2003) Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells. *J Exp Med* 198:1119–1126.
18. Bouillet P, et al. (2002) BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 415:922–926.
19. Pellegrini M, et al. (2004) Loss of Bim increases T cell production and function in interleukin 7 receptor-deficient mice. *J Exp Med* 200:1189–1195.
20. Hildeman DA, et al. (2002) Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity* 16:759–767.
21. Huang Q, Petros AM, Virgin HW, Fesik SW, Olejniczak ET (2003) Solution structure of the BHRF1 protein from Epstein-Barr virus, a homolog of human Bcl-2. *J Mol Biol* 332:1123–1130.
22. Loh J, et al. (2005) A surface groove essential for viral Bcl-2 function during chronic infection in vivo. *PLoS Pathog* 1:e10.
23. Taichman RS, Torigoe T, Tanaka S, Miyashita T, Reed JC (1992) Gene transfer investigations of p56-LCK function in IL-2-dependent T-cell lines: Implications for mechanisms of IL-2-signal transduction. *Cytokine* 4:441–453.
24. Watson J (1979) Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J Exp Med* 150:1510–1519.
25. Stahl M, et al. (2002) The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol* 168:5024–5031.
26. Zhu Y, et al. (2004) Constitutive association of the proapoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells. *Proc Natl Acad Sci USA* 101:7681–7686.
27. Esposti MD, Hatzinisiriou I, McLennan H, Ralph S (1999) Bcl-2 and mitochondrial oxygen radicals. New approaches with reactive oxygen species-sensitive probes. *J Biol Chem* 274:29831–29837.
28. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ (2003) Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J Biol Chem* 278:18811–18816.
29. Gavathiotis E, et al. (2008) BAX activation is initiated at a novel interaction site. *Nature* 455:1076–1081.
30. Hinds MG, et al. (2007) Bim, Bad and Bmf: Intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets. *Cell Death Differ* 14:128–136.
31. Bouillet P, Strasser A (2002) Bax and Bak: Back-bone of T cell death. *Nat Immunol* 3:893–894.
32. Rathmell JC, Lindsten T, Zong WX, Cinalli RM, Thompson CB (2002) Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nat Immunol* 3:932–939.
33. Zhu Y, et al. (2006) Bax does not have to adopt its final form to drive T cell death. *J Exp Med* 203:1147–1152.
34. Wasilenko ST, Banadyga L, Bond D, Barry M (2005) The vaccinia virus F1L protein interacts with the proapoptotic protein Bak and inhibits Bak activation. *J Virol* 79:14031–14043.
35. Taylor JM, Quilty D, Banadyga L, Barry M (2006) The vaccinia virus protein F1L interacts with Bim and inhibits activation of the pro-apoptotic protein Bax. *J Biol Chem* 281:39728–39739.
36. Marrack P, Kappler J, Mitchell T (1999) Type I interferons keep activated T cells alive. *J Exp Med* 189:521–530.