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Herpesvirus Saimiri Encodes a Functional Homolog of the Human *bcl-2* Oncogene

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Here we demonstrate that open reading frame 16 (ORF16) of the oncogenic herpesvirus saimiri protects cells from heterologous virus-induced apoptosis. The BH1 and BH2 homology domains are highly conserved in ORF16, and ORF16 heterodimerizes with Bcl-2 family members Bax and Bak. However, ORF16 lacks the core sequence of the conserved BH3 homology domain, suggesting that this region is not essential for anti-apoptotic activity. Conservation of a functional *bcl-2* homolog among gammaherpesviruses suggests that inhibition of programmed cell death is important in the biology of these viruses.

The gammaherpesviruses have been subdivided into two genera. The prototype gamma 1 herpesvirus (lymphocryptovirus) is Epstein-Barr virus (EBV), a human B-cell-tropic virus that is believed to have a prominent role in the etiology of a wide variety of human malignancies (47). EBV infection of B cells in culture leads to growth transformation and the establishment of a latent virus infection. Several EBV genes that are expressed during latency are required for growth transformation of lymphocytes in vitro, including EBNA-LP, -2, -3A, -3C, and LMP-1 (27). The prototype gamma 2 herpesvirus (rhadinovirus) is herpesvirus saimiri (HVS), a T-cell-tropic virus that rapidly induces fatal lymphomas in experimental infections of primates and rabbits (14). Although HVS does not encode homologs of the EBV EBNAs or LMP-1 that are required for growth transformation by EBV, HVS encodes the Stp oncogene that is required for cellular transformation and is not found in the EBV genome (25, 29, 44). HVS also encodes Tip (tyrosine kinase interacting protein) and homologs of the interleukin-8 (IL-8) receptor, cyclin D, and a superantigen that may also contribute to oncogenesis (1, 23, 24, 37, 56).

In addition to the latent genes required for growth transformation, EBV also encodes a homolog of the human *bcl-2* oncogene (8). *bcl-2* was first identified at t(14;18) translocations that occur in follicular B-cell lymphomas (8, 16, 52). This translocation event results in overexpression of *bcl-2* which allows B cells to survive when they would normally die by programmed cell death (41, 54). Overexpression of *bcl-2* in transgenic mice protects a variety of cell types from naturally occurring cell death (40). Transfection of a variety of cultured cell lines with *bcl-2* also protects cells from multiple death-inducing stimuli, including serum or growth factor withdrawal, treatment with calcium ionophores, glucose withdrawal, membrane peroxidation, and virus infection (2, 32, 38, 53, 60). The *bcl-2* gene also facilitates transformation by c-*myc* (3, 54). In the absence of *bcl-2*, the c-*myc* proto-oncogene induces apoptosis rather than transformation in serum-starved cells (3). The idea that inhibitors of apoptosis are important for cell transformation is a repeating theme, and the c-*myc* translocations observed in EBV-associated Burkitt's lymphoma suggest a potential need for an apoptosis inhibitor. However, the *bcl-2* homolog in EBV, BHRF1, is not required for growth transformation of B lymphocytes following infection in vitro, even though it possesses potent anti-apoptotic activity (20, 30, 39, 50). Furthermore, BHRF1 protein has not been detected in latently infected B cells but is predominantly expressed at early times following reactivation to the lytic virus replication cycle (13). Thus, BHRF1 has been postulated to have a role in blocking cell death during the lytic cycle. A number of viruses encode inhibitors of apoptosis, apparently to prevent premature cell death triggered by virus infection, allowing the virus to complete its replication cycle and produce abundant progeny before the cell dies (9, 10, 55).

When the HVS genome was originally sequenced, a *bcl-2* homolog was not detected (1). Subsequently, we and others noted significant amino acid homology between an open reading frame (ORF16) of HVS and a conserved domain found in members of the Bcl-2 protein family (49). Alignment of the amino acid sequence of ORF16 with that of several Bcl-2 family members indicated that while ORF16 shares colinear homology with the Bcl-2 family, significant amino acid identity is limited to the conserved BH1 and BH2 homology domains (Fig. 1A). The BH1-BH2 region was demonstrated previously to be important for protein-protein interactions between Bcl-2 family members and for anti-apoptotic function of Bcl-2 and Bcl-x_L (5, 48, 57).

A third, more recently described homology domain, BH3, is present in the cellular proteins included in Fig. 1 but is poorly conserved in ORF16. BH3 appears to be important for the death-inducing function of the Bcl-2-related proteins Bax and Bak (7, 59). Interestingly, sequences flanking the BH3 region are better conserved between ORF16 and other homologs. Based on the crystal structure of Bcl-x_L, the BH3 homology domain is located in helix 2, which is in close proximity to the helix-loop-helix structures of the BH1 and BH2 domains (43). The BH3 domain of Bax and Bak is required to form heterodimers with Bcl-x_L and is critical for the death-promoting activities of Bax and Bak (7, 58). Furthermore, a 46-amino-acid peptide containing the BH3 domain of Bak is sufficient to bind Bcl-x_L and kill cells (7). The role of BH3 in Bcl-2 and Bcl-x_L is not clear, and the striking absence of the highly conserved core

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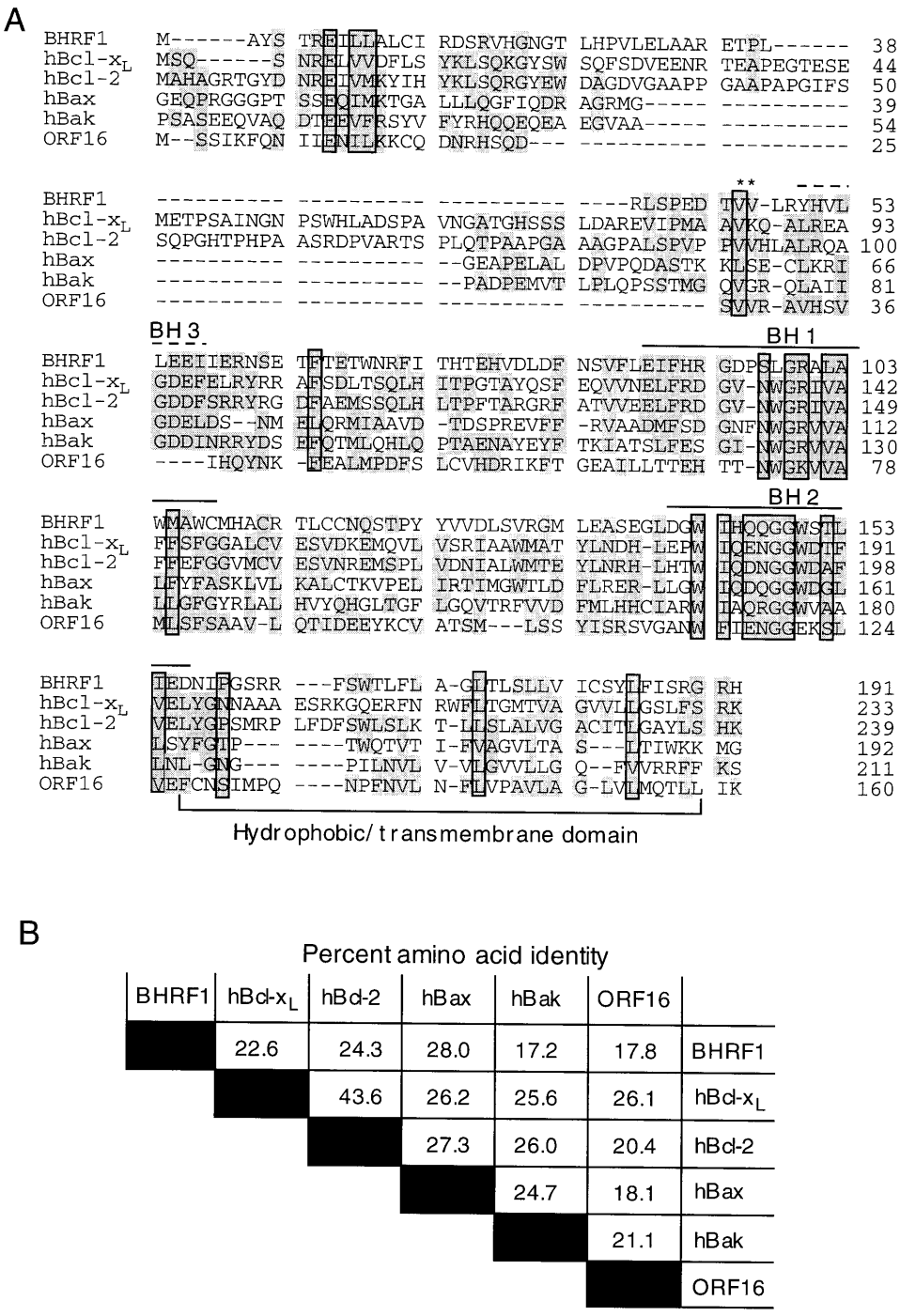

FIG. 1. Analysis of ORF16 amino acid sequence. (A) Alignment of the predicted HVS ORF16 amino acid sequence with the human proteins Bcl-x_L, Bcl-2, Bax, and Bak and the EBV homolog BHRF1. The BH1 and BH2 homology domains are indicated by horizontal lines and the BH3 domain by a dashed line. Mutations of starred residues in BHRF1 alter the interaction of BHRF1 with R*ras*. The putative transmembrane domain of ORF16 is bracketed. Black boxes indicate amino acid similarity. (B) Percent amino acid sequence identity between various members of the Bcl-2 family.

domain of BH3 in ORF16 suggests functional differences between ORF16 and its cellular homologs. ORF16 also contains a hydrophobic domain at its C terminus, suggesting that the ORF16 protein is localized to intracellular membranes as are other Bcl-2 family members (18, 42). The overall amino acid sequence identity between ORF16 and other Bcl-2 family members is low (17 to 26%) but within the range of identities observed for most family members (Fig. 1B).

We demonstrated previously that Sindbis virus kills a variety of cell types by inducing programmed cell death. Cells dying from Sindbis virus exhibit the morphological and biochemical characteristics of apoptosis including membrane blebbing,

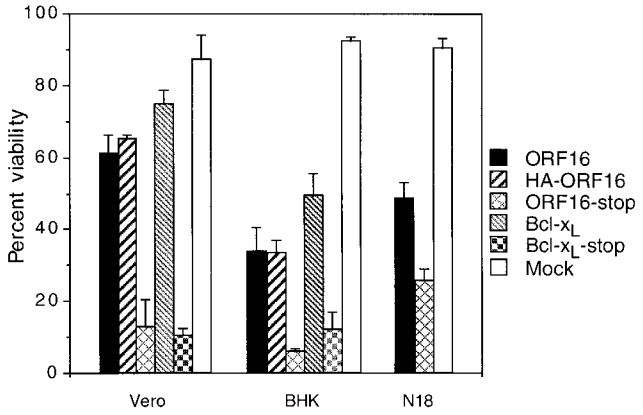

FIG. 2. ORF16 inhibits cell death. Cell viability was determined by trypan blue exclusion at 48 to 65 h postinfection with recombinant Sindbis viruses encoding the indicated genes. ORF16-stop and Bcl-x_L-stop virus constructs have stop codons inserted into their respective open reading frames. Two independent clones of both dsSV-ORF16 and dsSV-HA-ORF16 were tested. Data from 3 to 12 independent experiments (± standard errors of the mean) are presented, and results with the Bcl-2 homologs are statistically significant versus the stop-codon controls as determined by the Mann-Whitney U test ($P > 0.01$).

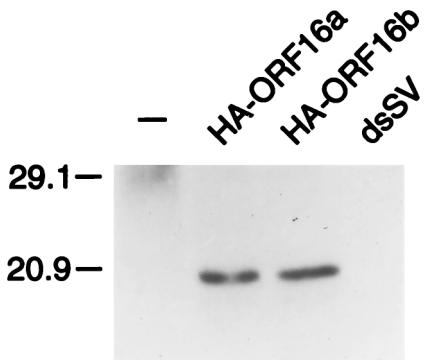

FIG. 3. Western blot demonstrating ORF16 protein expression by Sindbis virus. Cell lysates prepared from BHK cells infected with two independently derived dsSV-HA-ORF16 viruses were immunoblotted with anti-HA antibody. Molecular mass markers (kDa) are at left.

chromatin condensation, and DNA ladder formation (32, 33, 35, 53). To study the function of genes that regulate cell death, heterologous genes are expressed from the Sindbis virus genome and tested in a cell viability assay for the ability to modulate Sindbis virus-induced cell death. Using this system, we have demonstrated the anti- and pro-apoptotic function of a variety of cellular and viral genes (5, 12, 31). To determine if ORF16 has anti-apoptotic activity similar to that of Bcl-2 and Bcl-x_L or pro-apoptotic activity similar to that of Bax and Bak, ORF16 was analyzed using the Sindbis virus vector system. The open reading frame was amplified by PCR from the *Eco*RI "F" fragment of the HVS genome (provided by John Nicholas) and inserted into the double subgenomic Sindbis virus vector (dsSV), and recombinant Sindbis viruses were generated as previously described (5, 31).

Vero cells used to study HVS gene expression (45) were infected with recombinant Sindbis virus encoding ORF16 at a multiplicity of infection of 5 PFU per cell, and cell viability was determined 48 to 65 h later by trypan blue exclusion. ORF16 protected cells from virus-induced cell death almost as efficiently as Bcl-x_L (Fig. 2). Similar results were obtained with BHRF1 (data not shown). The HA-tagged version of ORF16 was equivalent to the unmodified protein in its ability to block cell death. In contrast, a stop codon inserted into the ORF16 coding sequences (*Spe*I amber codon linker from New England BioLabs inserted at the *Xba*I site in dsSV-ORF16) abolished all protective effects. This control demonstrated that the virus vector used to express ORF16 was competent to kill cells and that the death inhibitory function was derived from the ORF16 reading frame. Similar but less dramatic effects were obtained with BHK (baby hamster kidney) and N18 (mouse neuroblastoma) cells. These results demonstrate that ORF16 is a potent inhibitor of programmed cell death.

To verify expression of ORF16 protein, lysates were prepared from cells 16 h postinfection with the Sindbis vector expressing HA-tagged ORF16 and immunoblotted with 12CA5 anti-HA antibody (Berkeley). A 21-kDa protein was detected with two independently derived HA-ORF16 viruses, while no ORF16 protein was detected in cells infected with the virus vector alone (Fig. 3). The predicted size of ORF16 is 18 kDa, and the difference in migration is presumably accounted for by the HA tag.

Bax and Bak can block the protective effects of Bcl-2 and Bcl-x_L apparently through heterodimerization (15). To further study the function of ORF16 protein we sought to identify which cellular proteins directly interact with the viral homolog. Glutathione *S*-transferase (GST)–ORF16 fusion protein was tested for the ability to heterodimerize with ^{35}S-labeled in vitro-translated human Bcl-2, human Bcl-x_L, rat Bcl-x_S, human Bax, and HA-tagged human Bak. Approximately 5 μg of GST-ORF16 protein was bound to 30 μl of glutathione agarose beads (Sigma) and incubated with 300 μl of a solution containing 140 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM sodium orthovanadate, 50 mM Tris-HCl (pH 8.0), and 1 mg of bovine serum albumin per ml. In vitro-translated, ^{35}S-labeled Bcl-2-related proteins (5 to 10 μl) were added to the beads, and the mixture was rocked at room temperature for 1 h and then washed 3 times with a solution of 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 20 mM Tris-HCl (pH 8.0). The captured Bcl-2 family members were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography (Fig. 4A). ORF16 formed detectable dimers with itself and with Bcl-2, Bax, and Bak but failed to interact with Bcl-x_L or a spliced variant, Bcl-x_S. Similar amounts of the individual labeled proteins were used in this assay (data not shown).

A similar assay to that described for ORF16 was performed with the Bcl-2 homolog from EBV (Fig. 4B). Like ORF16, GST-BHRF1 formed dimers with itself, Bcl-2, and Bak. In contrast to ORF16, BHRF1 formed complexes with both long and short forms of Bcl-x but failed to bind Bax. The lack of interaction between BHRF1 and Bax is consistent with immunoprecipitation experiments reported by Theodorakis et al. (51). The same group observed BHRF1-Bak interactions in yeast two-hybrid studies (51), which is also consistent with our results. Bcl-2 and Bak failed to bind GST-herpes simplex virus VP16, verifying that Bcl-2 and Bak (the only proteins that bound to both GST-ORF16 and GST-BHRF1) did not nonspecifically bind to GST fusion proteins (data not shown).

Studies on Bcl-2 and Bcl-x_L demonstrate that the BH1 domain is required for heterodimerization with Bax and Bak (5, 48, 57). Because BHRF1 fails to bind with Bax, it could potentially escape negative regulation by Bax and represent a powerful constitutive death suppressor. Although BHRF1 binds to Bak, Bak may not function to inhibit BHRF1 because Bak appears to have anti-death rather than pro-death activity

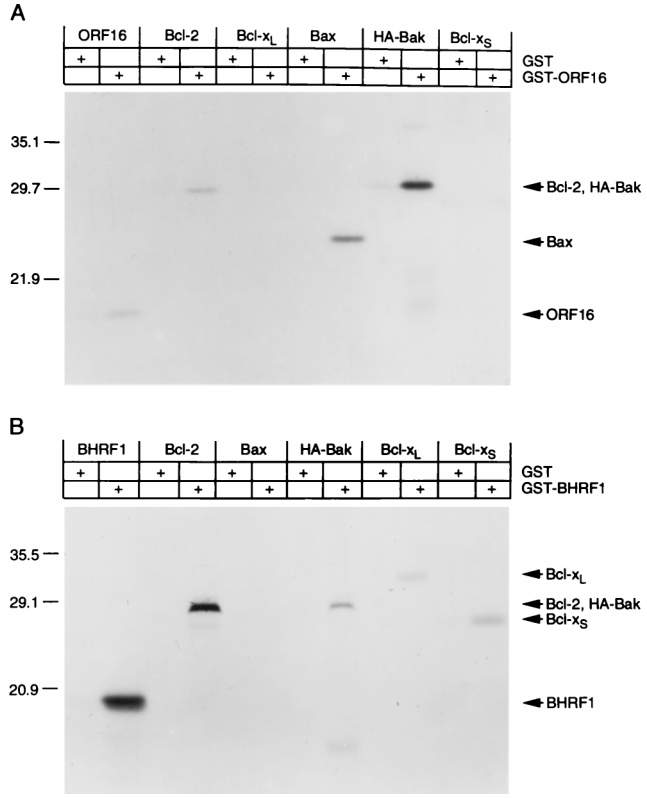

FIG. 4. GST pull-down assay to identify Bcl-2 family members that heterodimerize with ORF16 and BHRF1. GST-ORF16 (A) or GST-BHRF1 (B) proteins were mixed with ^{35}S-labeled in vitro-translated Bcl-2 family members. Labeled proteins that bound to the GST fusion proteins or to GST alone were analyzed on 15% polyacrylamide gels. The HA tag on Bak does not appear to alter function or dimerization (5). Molecular mass markers (kDa) are at left.

in EBV-positive B cells (26). In contrast, ORF16 binds to both Bax and Bak, although it is not known if these cellular pro-death proteins alter the function of ORF16.

Bcl-2 homologs were also recently identified in the gamma 2 herpesviruses bovine herpesvirus 4 (36) and Kaposi's sarcoma-associated herpesvirus (6). The presence of functional *bcl-2* homologs in both gamma 1 (EBV) and gamma 2 (HVS) herpesviruses suggests that anti-apoptotic activity is important in some phase of the virus life cycle. The conservation of BHRF1 in natural isolates of EBV further supports this hypothesis (19). However, EBVs deleted for BHRF1 remain competent to replicate and to transform B cells in vitro (30, 39), suggesting the possibility that viral Bcl-2 may not have a direct role in tumorigenesis.

BHRF1 is a potent inhibitor of cell death and can cooperate with adenovirus E1A to facilitate transformation of primary fibroblasts (20, 50, 51). Although adenovirus transforms cells in culture, adenovirus infections have not been associated with tumors in humans. However, the link between transformation and anti-apoptotic function observed with adenovirus has interesting implications for the anti-apoptotic function of gammaherpesviruses. Interestingly, both HVS and Kaposi's sarcoma-associated herpesvirus encode a homolog of cyclin D (1, 4). Cellular cyclin D has been associated with both transformation and apoptosis (22, 28). Thus, an anti-apoptotic function may be required to counteract some of the pro-apoptotic effects of viral cyclin D. Any potential cooperation between ORF16 and other HVS-encoded genes such as the cyclin homolog or Stp has not been explored.

It has been suggested that BHRF1 protects cells from EBV-induced apoptosis, allowing the virus to complete its replication cycle prior to cell death (20). Others have suggested that BHRF1 has a role in tumorigenesis by blocking cell death either in the face of DNA damage (21) or in abortive lytic infections (11). BHRF1 is abundantly expressed in oral "hairy" leukoplakia, a lytic EBV infection of the tongue in AIDS patients characterized by epithelial thickening and hyperkeratosis similar to that observed with SCC12F cells overexpressing BHRF1 (11, 17). These observations draw a potential link between BHRF1 and the epithelial tumors associated with EBV infection such as nasopharyngeal carcinoma (34, 46). In contrast to EBV, HVS is much more amenable to study as it can be plaque titered and easily propagated in culture. In addition, there are animal models for HVS-induced tumorigenesis (14). Thus, HVS will provide an important system in which to study the role of anti-apoptotic genes in herpesvirus infection and tumorigenesis.

V.E.N. and E.H.-Y.C. contributed equally to this work.

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