Herpesvirus papio encodes a functional homologue of the Epstein–Barr virus apoptosis suppressor, BHRF1

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The human tumour virus Epstein–Barr virus (EBV) encodes a 17 kDa protein, BHRF1, which is a member of the Bcl-2 family and has been shown to suppress apoptosis. The role of this gene in the life-cycle of EBV has not been fully elucidated. In order to identify motifs conserved in herpesviruses and possibly shed light on its function we isolated a BHRF1 homologue from herpesvirus papio (cerco-pithecine herpesvirus-12) a closely related gammaherpesvirus of baboons. The gene, hvpBHRF1, also encodes a 17 kDa protein which shares 64% identity and 79% similarity with EBV BHRF1 at the amino acid level. In biological assays, hvpBHRF1 and BHRF1 conferred similar levels of protection on human keratinocytes induced to apoptose with cis-platin.

Greater than 90% of the human population are infected for life with Epstein–Barr virus (EBV) and a similar percentage of captive baboons are seropositive for herpesvirus papio (HVP) (Jenson et al., 2000). DNA sequence analysis and studies on genome organization and gene function indicate that EBV is more closely related to HVP than any other human herpesvirus (Heller & Kieff, 1981; Heller et al., 1981; Ryon et al., 1993; Yates et al., 1996). Both belong to the lymphocryptovirus subfamily of gammaherpesviruses. Many of the EBV genes associated with latency and biological features such as the ability to infect and growth transform B cells are conserved (Falk et al., 1977; Moghaddam et al., 1998). EBV genes like the EBERs (Toczyski & Steitz, 1993), EBNA2 (Dawson et al., 1992), the LMPs (Franken et al., 1995, 1996) and EBNA1 (Yates et al., 1996) have equivalents in HVP. Both the latency-associated origin of replication, oriP, and the origin of lytic replication, oriLyt, are conserved (Loeb et al., 1990; Ryon et al., 1993). There is also a high degree of conservation of transcriptional control elements including the promoters used in latency and those around the origin of lytic replication (Ryon et al., 1993; Fuentes-Panana et al., 1999).

BHRF1, a 17 kDa protein, is expressed during the switch from latency to lytic replication, is found in all strains of EBV thus far analysed and is conserved at both the sequence and functional levels among EBV strains (Khanim et al., 1997). However, studies with BHRF1 deletion mutants showed that the gene is not essential for virus replication or for the transformation of B cells in vitro (Marchini et al., 1991; Lee & Yates, 1992). Cleary et al. (1986) reported that BHRF1 shares distant sequence homology with the human proto-oncogene Bcl-2, which is associated with the t(14;18) chromosomal translocation found in follicular cell lymphoma (Tsujimoto et al., 1984), and suppresses apoptosis in cells (Vaux et al., 1988; Hockenbery et al., 1993). There is compelling evidence that BHRF1 also suppresses apoptosis and is thus a functional homologue of Bcl-2. BHRF1 can block both Fas- and TNF-mediated apoptosis (Kawanishi, 1997; Foghsgaard & Jaattela, 1997) and apoptosis induced with stimuli such as cis-platin and ionomycin (Henderson et al., 1993; Khanim et al., 1997). Furthermore, apoptosis induced by growth factor withdrawal (Henderson et al., 1993; Dawson et al., 1995, 1998), p53 or c-myc expression (Theodorakis et al., 1996; Fanidi et al., 1998) is in all cases inhibited by BHRF-1.

BHRF1 shares only distant sequence homology (about 25%) with human Bcl-2, with similarity residing mainly in the BH1 and BH2 domains, and the carboxyl-terminal end. It was reasoned that sequences conserved between gammaherpes-virus genes may be important in gene function and their identification would allow the design of mutations that elucidate BHRF1 function. The 7 kbp XbaI I fragment of HVP (Ryon et al., 1993) hybridized extensively with the BamHI H fragment of the B95-8 strain of EBV and probably contained the HVP BHRF1 homologue. The 3’ end of the HVP clone was sequenced to a redundancy of at least 3 by primer walking with overlapping forward and reverse primers. An ORF in the 1·1 kbp sequence obtained had extensive homology to BHRF1; however, it had no translation termination sequence, apparently finishing in the adjacent Xbal fragment (XbaI L) of...
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Fig. 1. Similarity of the EBV and HVP BHRF1 ORFs to EBV ORF BALF-1, HHV-8 orf16 and the cellular anti-apoptotic gene Bcl-2.

Predicted amino acid sequences (shown in single-letter code) were aligned via the PIMA multiple sequence alignment program, using the sequential branching clustering method. Areas of identity are shown in black; conserved regions are shown in grey.

The major regions of conservation in other Bcl-2 family members, BH1–4, are indicated above the aligned sequences.

HVP. An HVP forward primer (5’ acgtttatggaggcagctt 3’) designed from a region of the ORF having minimal homology to BHRF1 (to avoid the possibility of amplifying EBV) and a reverse primer (5’ ggcatgttattcttgtaagc 3’) based in the coordinates 55119–55100 of the B95-8 strain of EBV (which is 3’ of the EBV BHRF1 ORF) were used to amplify the 3’ end of the HVP ORF from HVP genomic DNA. The purified product was sequenced twice (without cloning) and the data merged with the initial sequence obtained from pJR7. 600 bp of the HVP sequence (GenBank acc. no. AF120456) was aligned with the BHRF-1 from the B95-8 strain of EBV. Although there are two ATGs, 76 bp apart, at the 5’ end of the HVP sequence, only the second ATG conforms with the Kozak consensus sequence for translational initiation (Kozak, 1986). The HVP protein showed 64% identity and 79% similarity with BHRF1. This similarity runs through the entire length of the two polypeptides. BHRF1 has two potential N-glycosylation sites which are not used (Pearson et al., 1987), and are not found in the HVP sequence. An alignment of hvpBHRF1 with several Bcl-2 homologues is shown in Fig. 1. Both the BH1 and BH2 domains, which are found in many species of the Bcl-2 family, are conserved between BHRF1 and the HVP sequence, both in key residues and in position. The hydrophobic carboxy-terminal end with the basic pentapeptide sequence downstream is also well conserved between the two. The amino-terminal regions of the EBV and HVP molecules are conserved, although comparison with Bcl-2 over the BH4, BH3 and intervening loop region reveals poor homology, possibly reflecting the loss of regulatory regions.

Among the Bcl-2 family (Cleary et al., 1985; Pearson et al., 1987; Henderson et al., 1993), the degree of homology varies significantly. Most viral homologues are only distantly related to Bcl-2, with percentage identity ranging from 15–30% at the amino acid level. hvpBHRF1, for example, has about 25% homology with Bcl-2 at the amino acid level and lower homology with other viral homologues like LMW5-HL (A179L) of African swine fever virus and the adenoviral E1B19K protein [for reviews see Teodoro & Branton (1997); Young et al. (1997)]. The degree of identity between hvpBHRF1 and other Bcl-2 homologues recently isolated from
other herpesviruses (ORF 16 in herpesvirus saimiri and v-Bcl2 in human herpesvirus-8; Nava et al., 1997; Sarid et al., 1997; Cheng et al., 1997; reviewed by Kieff & Shenk, 1998) is less than 20%.

Having identified the HVP ORF, it was cloned from HVP genomic DNA while EBV BHRF1 was subcloned from the EcoRI A restriction fragment of the B95-8 strain. PCR was used to amplify the two ORFs from the appropriate templates; the products were cloned in pT7Blue (Novagen) and subcloned in the EcoRI–BglII site of the eukaryotic expression vector pSG5 (Stratagene) as pSGBHRF1 and pSGBcl2 respectively. All clones were sequenced to ensure authenticity.

To determine whether the hvpBHRF1 can be expressed and to estimate the size of the encoded protein (the predicted size is approximately 21 kDa), we used the TnT T7 coupled in vitro transcription/translation kit to obtain the two proteins labelled with [35S]methionine. Fig. 2(a) shows that hvpBHRF1 appears as an approximately 17 kDa protein in SDS–PAGE gels, similar in size to BHRF1. The discrepancy in the size of BHRF1 (predicted molecular mass is 21 kDa) has been attributed to anomalous migration in gels rather than any post-translational modification (Austin et al., 1988). In immunoprecipitation assays, hvpBHRF1 was not recognized (not shown) by either an anti-BHRF1 monoclonal antibody, 5B11 (Chemicon) (Pearson et al., 1987), or by an anti-BHRF1 polyclonal antibody, DE97. This polyclonal antibody specifically immunoprecipitates EBV BHRF1 (Meseda, 1999) and was raised by immunizing rabbits with a purified maltose binding protein–BHRF1 fusion (MBP–BHRF1) [made by cloning a RsaI–BamHI fragment of BHRF1 (co-ordinates 54444–54858) in-frame with the malE gene in the pMALc2 vector (New England Biolabs) and expressing in E. coli]. Thus despite the high degree of identity between BHRF1 and hvpBHRF1 the major antigenic sites of the proteins are not well conserved.

In order to be able to detect hvpBHRF1 expression, it was tagged in-frame with the nine amino acid haemagglutinin (HA) epitope (YPYDVPDYA) of influenza virus. To assess the suitability of the haemagglutinin epitope tag, hvpBHRF1-HA was expressed by in vitro transcription/translation and immunoprecipitated with an anti-HA monoclonal antibody, 12CA5 (Boehringer Mannheim). Fig. 2(b) shows hvpBHRF1-HA as an approximately 17–18 kDa protein which was immunoprecipitated by the HA-specific monoclonal antibody (12CA5) from the translation product. Both BHRF1 and Bcl2 included in the assay were precipitated with their respective antibodies. The pSG5 vector control and pSGhvpBHRF1 immunoprecipitated with anti-HA antibody were negative, showing that the product in lane 4 was specifically recognized by monoclonal antibody 12CA5. SV-K cells transfected with pSGhvpBHRF1-HA were also detectable by indirect immunofluorescence staining at 48 h post-transfection, using monoclonal antibody 12CA5 as primary antibody. The subcellular localization of hvpBHRF-1 in SV-K cells was analysed by immunofluorescence (data not shown) and was similar to that reported for EBV BHRF-1 and Bcl-2, being localized to the mitochondrial and nuclear membranes (Hickish et al., 1994; Dawson et al., 1995).

Ectopic expression of BHRF1 in cells has been shown to protect a wide variety of cells, including epithelial and B cells, from apoptosis induced by a wide range of stimuli (reviewed by Shen & Shenk, 1995; Teodoro & Branton, 1997; Young et al., 1997). cis-Platin is a drug used in cancer therapy and has been shown to cause DNA damage in cells, thereby provoking signalling leading to cell death by apoptosis (Sorenson et al., 1990; Lowe et al., 1993; Dawson et al., 1995). Two epithelial cell lines, SCC12F (squamous cell carcinoma) and SV-K cells (derived from human keratinocytes immortalized with the large T antigen of SV40), can be readily induced into apoptosis by cis-platin. BHRF1 expression in these cell lines protected them from cis-platin-induced apoptosis (Dawson et al., 1995; Eliopoulos et al., 1996). To assess whether hvpBHRF1 and the tagged gene (hvpBHRF1-HA) are biologically active, SV-K cells were transfected separately with the plasmids pSGhvpBHRF1-HA, pSGhvpBHRF1, pSGBHRF1, pSGBcl2

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**Fig. 2** In vitro expression of haemagglutinin-tagged hvpBHRF1. (a) Crude [35S]-labelled translation products, with the tagged protein at about 17.5–18 kDa. (b) Tagged protein (lane 4) immunoprecipitated with the anti-HA monoclonal antibody 12CA5. Both BHRF1 (lane 2) and Bcl-2 (lane 7) were included as positive controls and immunoprecipitated with their specific monoclonal antibodies, 5B11 and 12CA5 respectively. The pSG5 vector was included as a negative control and immunoprecipitated with 5B11 (lane 5) and 12CA5 (lane 6). Proteins were resolved in 11% SDS–PAGE gels, followed by autoradiography. The molecular masses of protein markers run in parallel are indicated.
and pSG5. Expression of proteins in the transfected cells was confirmed by indirect immunofluorescence (data not shown). The transfected cells were tested for viability following exposure to a range of concentrations of cis-platin. Fig. 3 shows that transfected cells were protected from cis-platin-induced toxicity. For example, at a drug concentration of 50 μM, 88, 84, 71 and 67% of cells (transfected with BHRF1, hvpBHRF1, hvpBHRF1-HA and Bcl-2 respectively) survived, as compared to 48% in the vector control. The tagged protein showed a relatively lower level of protection when compared with the untagged hvpBHRF1. This observation is similar to an earlier report (Hockenbery et al., 1993), where a deletion of the transmembrane domain from Bcl-2 reduced its ability to protect a murine myeloid cell line from apoptosis. Since the transmembrane domain is thought to anchor Bcl-2-related proteins to membranes where they localize, the observation here suggests that manipulation of this domain can reduce the ability of this family of proteins to protect cells from induced death, but does not totally abrogate their function. It was also noted that cells expressing the untagged hvpBHRF1 or BHRF1 showed a relatively higher percentage of surviving cells than cells expressing Bcl-2. Thus, the Bcl-2 homologue in HVP is not only a structural homologue of the EBV BHRF1, but also a functional homologue.

Several Bcl-2 homologues have been isolated from a wide range of organisms including other viruses, and the products are either apoptosis inducers or suppressors. Interestingly a second functional anti-apoptotic gene related to Bcl-2, encoded by the BARF1 ORF, has recently been identified in EBV (Marshall et al., 1999), thus further highlighting the importance of the control of apoptosis in the life-cycle of herpesviruses.

It is widely speculated that expression of BHRF1 prolongs the life-span of cells in which EBV is actively replicating thereby enhancing virus production. This argument is lent credence by the abundant expression of BHRF1 in oral hairy leukoplakia, which is believed to be a focus of active EBV replication. hvpBHRF1 is the most similar to BHRF1 among Bcl-2 homologues known to date. Since hvpBHRF1 showed a similar biological activity to BHRF1 in our assay, it provides a more relevant homologue for a comprehensive structural/functional analysis of the proteins. Furthermore, the high degree of similarity between HVP and EBV, as reported here and elsewhere, make it a good candidate for comparative analysis of EBV genes, possibly providing a primate in vivo model for EBV and an indication of important epitopes in EBV proteins.

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References


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