Kaposi's sarcoma-associated herpesvirus encodes a functional Bcl-2 homologue

RONIT SARID[1], TAKAAKI SATO[1,2], ROY A. BOHENZKY[1], JAMES J. RUSSO[3] & YUAN CHANG[1]

[1]*Department of Pathology, Columbia University College of Physicians & Surgeons*
[2]*Department of Otolaryngology, Columbia University College of Physicians & Surgeons*
[3]*Columbia Genome Center, Columbia University. 630 West 168th Street, New York, New York 10032*
Correspondence should be addressed to Y.C.

Kaposi's sarcoma-associated herpesvirus (KSHV) is a newly discovered herpesvirus etiologically associated with Kaposi's sarcoma (KS) and two lymphoproliferative disorders. We describe a KSHV v*bcl-2* gene with homology to the proto-oncogene *bcl-2*. It is expressed in KS lesions and in cell lines derived from primary effusion lymphomas. Using yeast and human cells we demonstrate the ability of KSHV vBcl-2 protein to suppress Bax toxicity. We show that KSHV vBcl-2 heterodimerizes with human Bcl-2 in a yeast two-hybrid system. These results suggest that KSHV vBcl-2 plays an anti-apoptotic role in virus infected cells.

KSHV is a gamma-2 herpesvirus[1] associated with all clinical subtypes of KS[2-7]. Cumulative data from epidemiologic and serologic studies indicate that infection by KSHV preceeds development of KS, is not common in the general population and is predominantly found in populations at high risk for developing KS[8-13]. KSHV is also found in primary effusion lymphomas (PEL)[14] and AIDS-related multicentric Castleman's disease (MCD)[15], two lymphoproliferative disorders with a known association to KS[16,17]. Although the repertoire of virus-cell interactions in these KSHV related disorders is just beginning to be investigated, viral strategies which allow the organism to escape from host responses appear essential. A number of DNA viruses including herpesvirus saimiri (HVS), Epstein Barr virus (EBV), African swine-fever virus (ASFV) and adenovirus, carry apoptosis-inhibiting genes which may allow these viruses to escape apoptotic host response during productive and/or persistant infection[18-21].

The cellular *bcl-2* gene has been shown to be involved in the regulation of apoptosis, a process of programmed cell death[22,23]. This gene is further implicated in the pathogenesis of human follicular lymphomas[24,25] as well as a wide variety of other human cancers. Dysregulated expression of *bcl-2* in such cases is believed to contribute to neoplastic cell expansion via an anti-apoptotic effect which enhances cell survival rather than by accelerating rates of cellular proliferation. Although the mode by which Bcl-2 affects the process of cell death is unknown, recent studies indicate that the Bcl-2 protein binds to other proteins with which it shares amino-acid sequence homology, including Bax, Bcl-X-L, Bcl-X-S, Mcl-1, Bik and Bad[26-29]. The functional significance of many of these Bcl-2 family protein-protein interactions remains enigmatic, however the heterodimerization of Bcl-2 with Bax appears crucial in preventing Bax mediated apoptosis[26]. Bax appears to antagonize Bcl-2 function, abrogating the ability of Bcl-2 to prolong cell survival. It is not clear which of these two proteins, Bcl-2 or Bax, is the active effector and which is the regulator. Bcl-2 could induce a pathway that actively maintains cell survival, with Bax serving as a negative regulator of Bcl-2, or alternatively, Bax could directly or indirectly generate death signals, with Bcl-2 serving as a dominant inhibitor of Bax. Here we report the identification of a virally encoded *bcl-2* homologue, KSHV v*bcl-2*, and demonstrate the function of the protein suggesting it is an important regulator of cell death.

KSHV v*bcl-2* sequence analysis

To identify the KSHV v*bcl-2*, a recombinant lambda phage (L36) was isolated from a lambda FIX II genomic library of a KSHV infected PEL cell line known as BC-1 (ref. 30) by cross hybridization to previously identified KSHV DNA sequences[31]. The 12.8 kb DNA insert of L36 was subcloned into M13mp18 (ref. 32) and sequenced. Sequences were assembled and potential open reading frames (ORFs) were identified. BLASTX alignment[33] of L36 identified a 525 bp ORF with a remarkable degree of similarity to the *bcl-2* proto-oncogene. The predicted protein encoded by KSHV v*bcl-2* is 175 amino acids in length, has an isoelectric point of 9.6 and predicted molecular mass of 19.4 kDa. Comparison of the entire protein sequence of KSHV vBcl-2 with that of human Bcl-2, Bcl-X-L and Bax revealed 58.7%, 60.2% and 62.3% conservation of amino acids, respectively (Fig.1). Furthermore, amino-acid similarity was detected to viral members of the Bcl-2 family: HVS ORF16 (65%); EBV BHRF1 (63.3%) and ASFV LMW5-HL (60.8%). Conserved BH1 and BH2 domains necessary for the formation of homo- and heterodimers among members of the Bcl-2 family were also identified in KSHV v*bcl-2* (Fig. 1). As in Bcl-2 and several other members of the Bcl-2 protein family, a C-terminal hydrophobic domain is present, indicating that KSHV vBcl-2 may be membrane-localized. This homology between KSHV vBcl-2 and other members of the Bcl-2 family suggested that KSHV vBcl-2 might also be functional in regulating apoptosis and enhancing cell survival in KSHV infected cells.

Expression of KSHV v*bcl-2* in infected cells

To determine whether KSHV v*bcl-2* is expressed in KSHV-related malignancies, we investigated the presence of its transcripts using reverse transcriptase-polymerase chain reaction (RT-PCR).

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KSHV    M-------------DE-D--VLP----------GEVLAI--EGIFM-ACGLN---------------EPE
HVS     M-------------SS-I--KFQ----------NIIENI--LKKCQ-DNRHS---------------Q-D
EBV     M-------------AY-STREIL---------LALCIR--DSRVH-GNGTL---------------HPV
ASFV    M-------------EG-E--ELI----------YHNI-I--NEILV-GYIKY---------------YMN
hBax    M-------------DGSG--E------QP----RGGGPTSSEQIMK-TGALLLQGFIQDRAGRMGGEAPE
hB-X-L  MSQS------NRELVV-D--FLSYKLSQKGYSWSQFSDV--EENRTEAPEGT---------------ESE
hBcl    MAHAGRTGYDNREIVM-K--YIHYKLSQRGYEWDAGDVG--AAPPGAAPAPG---------------IFS

KSHV    -----------YLYHPLL---SPIK--------LYI--TGLMR---DK-E-SLFEA--------MLA---
HVS     -----------SVVRAVH---SVIH--------QYN--K--------------FEA--------LMP---
EBV     -----------LELAARE---TPLR--------LSPEDTVVLR---YH-V-LLEEI--------IER---
ASFV    -----------DISEHEL---SPYQ--------QQI--KKILT---YYDE-CLNKQ--------VTI---
hBax    -----------LALDPVPQDASTKK--------LSE--C-------------LKRIGDELDSMNELQ---
hB-X-L  METPSAINGNPSWHLADS---PAVNGATGHSSSLDA--REVIPMAAVK-Q-ALREAGDEFELRYRRAFSD
hBcl    SQPGHTPHTAASRDPVAR---TSPLQTPAAPGAAAG--PALSPVPPVV-HLTLRQAGDDFSRRYRRDFAE

                                    BH1
KSHV    -N-VRF-----HSTTGI-NQL-GL---SMLQV--SGD-G-NMNWGRALAILTFGSFV-AQK-LSNEPHLR
HVS     -D-FSL-----CVHDRI-KFT-GE---AILLT--TEH---TTNWGKVVAMLSFSAAV-LQT--TDEEYKC
EBV     -NSETFTETWNRFITHT-EHV-DLDFNSVFLE--IFHRG-DPSLGRALAWMAWCMHA-CRT-LCCNQSTP
ASFV    -T-FSL-----TNAQEI-KTQ-FT---GVVTE--LFK-D-LINWGRICGFIVFSARM-AKY-CKDANNHL
hBax    -R-MIA-----AVDTDS-PRE-VF---FRVAADMFSD-G-NFNWGRVVALFYFASKL-VLKALCTKVPEL
hB-X-L  LT-SQL-----HITPGTAYQSFEQ---VVNEL--FRD-G--VNWGRIVAFFSFGGALCVES-VDKEMQVL
HBcl    MS-RQL-----HLTPFTARGRFAT---VVEEL--FRD-G--VNWGRIVAFFEFGGVMCVE-SVDKEMQVL

                                    BH2
KSHV    DFALAVLPV-YAYEAIGPQ--WFRARGGW-RGLKAYCTQV---LTRR---RGRRMTALLGSIALLATILA
HVS     V-ATSMLSS-YISRSVGAN--WFIENGGE-KSLVEFCNSI---MPQN---PFNVLNFLVP--AVLAGLVL
EBV     YYVVDLSVR-GMLEASEGLDGWIHQQGGW-STLIEDNIPG---SRRF---SWTLFLAGLTLSLLVICSYL
ASFV    ESTVITTAY-NFMKHNLLP--WMISHGGQ-EEFLAFSLHS---DIYS---VIFNIKYFLSKFCNHMFLRS
hBax    IRTIMGWTL-DFLRERLLG--WIQDQGGW-DGLLSYFG-------TP---TWQTVTIFVAG--VLTASLT
hB-X-L  VSRIAAWMATYLNDHLEP---WIQENGGWDTFVELYGNNAAAESRKG---QERFNRWFLTGMTVAGVVLL
hBcl    YDNIALWM-TEYLNRHLHT--WIQDNGGW-DAFVELYGPS---MRPLFDFSWLSLKTLL-SLALVGACIT

KSHV    AVA-MSR----R
HVS     MQT-LLI----K
EBV     FIS-RGR----H
ASFV    CVQ-LLRNCNLI
hBax    -IW-KKM----G
hB-X-L  GSL-FSR----K
hBcl    LGAYLGH----K
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Fig. 1 Amino-acid sequence and homology comparison of KSHV vBcl-2. HVS, EBV and ASFV refer to the HVS ORF16, EBV BHRF1 and African swine-fever virus LMW5-HL proteins, respectively. Bold type denotes amino acids that are identical in at least four of the proteins shown. BH1 and BH2 are conserved domains necessary for multimerization interactions among members of the Bcl-2 family. Hydrophobic C-terminal, putative membrane localization sequences are underlined and only LMW5-HL, the ASFV Bcl-2 homologue, lacks a putative hydrophobic transmembrane domain[51]. Sequences were aligned using the software ALIGN[52]. KSHV *vbcl-2* is identified as ORF16 within the KSHV genome[31] submitted to GenBank (U7569).

Total RNA was isolated from KS lesions of HIV seropositive and HIV seronegative North American and Italian patients. Total RNA was also obtained from two KSHV infected PEL cell lines, BC-1 and BCP-1. BC-1 is an EBV co-infected cell line[30] and BCP-1 is an EBV negative cell line that we derived from an HIV seronegative man with a history of KS and PEL[11]. The KSHV *vbcl-2* open reading frame transcript was detected in all KSHV infected specimens while it was absent in control B-cells infected only with EBV (Fig. 2). Northern analysis of KSHV *vbcl-2* mRNA using a strand-specific riboprobe reveals a 3.4 kb transcript expressed at low levels in normal growth conditions (Fig. 3, lane 1) and at high levels in cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Fig. 3, lane 2). A 0.7 kb transcript is detected in the TPA-induced cells. Each of the two transcripts have more than one start site as confirmed by isolation of cDNA clones which are undergoing further mapping and characterization.

Neutralization of Bax toxicity by KSHV vBcl-2

In yeast, expression of human Bax triggers acute cell death which can be prevented by Bcl-2 (ref. 27). The effect of KSHV vBcl-2 on yeast survival was investigated using a Bax suppression assay in which expression of Bax as a fusion protein with either an amino terminal DNA-binding or trans-activation domain results in a lethal phenotype that can be specifically suppressed by co-expression of Bcl-2 and other apoptotic repressors[27,34,35]. For these experiments, the full-length Bax protein was expressed as a fusion protein under the control of a strong constitutive ADH promoter in the plasmid pEG202. The KSHV vBcl-2 was expressed as a fusion protein containing the entire vBcl-2 sequence except for the C-terminal transmembrane domain, under the control of an inducible GAL1 promoter in the pJG4-5 plasmid. Yeast were transformed with pEG202-Bax and pJG4-5-KSHV *vbcl-2* expression plasmids. Half the cells were plated on glucose containing medium which does not induce the GAL1 promoter and the remainder of the transformed cells were plated on galactose containing medium which activates the GAL1 promoter. Numerous colonies formed on the galactose containing medium, whereas only a few

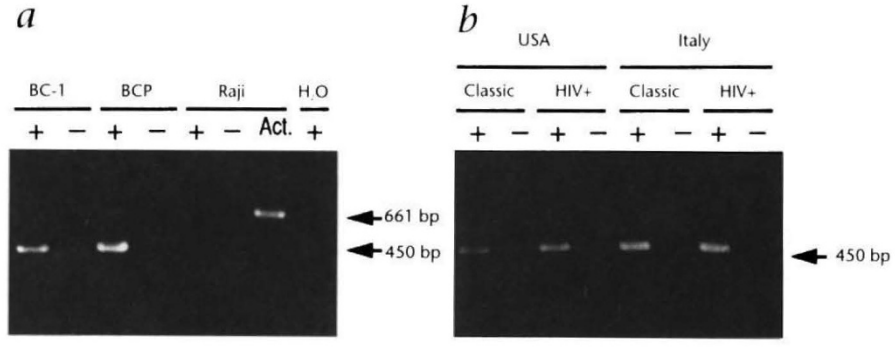

Fig. 2 Detection of the KSHV *vbcl-2* expression by RT-PCR. Expression of the KSHV *vbcl-2* open reading frame was examined by RT-PCR and identified as a 451 bp band. Expression was detected in RNA obtained from four KS specimens (HIV seropositive and HIV seronegative patients) and two PEL cell lines (BC-1: co-infected with KSHV and EBV; BCP-1: infected with KSHV only). Negative controls included RT-PCR performed on RNA in the absence of reverse transcription reaction (–) and with water only. RT-PCR was performed on RNA extracted from the KSHV negative/EBV positive B-cell line, Raji, and was negative for KSHV *vbcl-2* but positive for β-actin (661 bp).

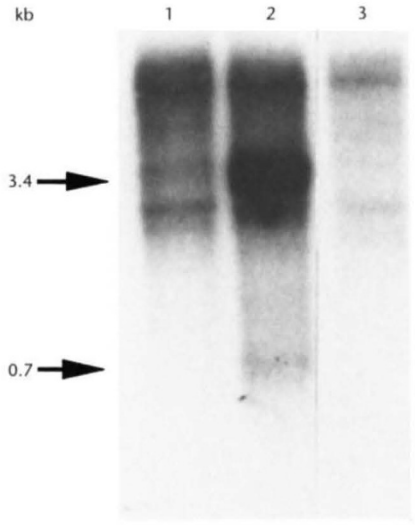

Fig. 3 Detection of KSHV *vbcl-2* expression in BC-1 cell line by northern blot hybridization. mRNA from BC-1 cell line (lane 1), TPA-induced BC-1 cell line (lane 2) and TPA-induced CB33 cell line infected with EBV (lane 3) was probed with a riboprobe representing the anti-sense mRNA of KSHV *vbcl-2*. Two major transcripts of 3.4 and 0.7 kb were detected in BC-1 induced with TPA while only a low level of the larger transcript was detected in non-induced BC-1. These transcripts were not detected in TPA-induced CB33.

Table 1 Yeast two-hybrid assay for KSHV vBcl-2 heterodimerization

		B42		
LexA	Medium	HSHV vBcl-2	huBcl-2	Lamin
huBcl-2	Galactose	+	++	–
	Glucose	–	–	–
Ras	Galactose	–	–	–
	Glucose	–	–	–

Versions of the pEG202 expression plasmids producing either LexA DNA-binding-domain fusion proteins (listed at left) or derivatives of pJG4-5 that encode B42 trans-activation-domain fusion proteins (listed at top) were introduced into 9015 yeast cells (3 μg each). The resulting transformants were grown on plates containing galactose/raffinose or glucose as a carbon source. Combinations of two-hybrid plasmids that resulted in galactose-dependent production of blue colored colonies are indicated as + or ++ depending on the relative strength of the blue color reaction. The positive combinations produced discernible blue color within two hours by filter assay. The converse experiment using a LexA/KSHV *vbcl-2* could not be performed because of non-specific reporter gene activation.

colonies grew on glucose plates, indicating that GAL1 induced expression of the pJG4-5-KSHV *vbcl-2* prevented Bax toxicity (Fig. 4*b*). The control protein B42/LaminC failed to abrogate the suppressive effects of LexA/Bax on colony formation (Fig. 4*c*).

Heterodimerization of vBcl-2 with human Bcl-2

The Bax suppression assay in yeast demonstrates the ability of KSHV *vbcl-2* to neutralize Bax toxicity but does not address whether KSHV vBcl-2 directly interacts with other members of the Bcl-2 family. A LexA/Bcl-2 fusion protein (containing the human Bcl-2 protein without the C-terminal transmembrane domain) resulted in trans-activation of the *lacZ* reporter gene (containing LexA operators), when coexpressed with a fusion protein representing the B42 trans-activation domain fused with KSHV vBcl-2. Furthermore, trans-activation of the lacZ reporter occurred only when cells were grown on galactose containing plates but not on glucose plates, consistent with the galactose dependency of the GAL1 promoter used to drive expression of the B42/KSHV *vbcl-2* expression plasmid. The interactions of KSHV vBcl-2 as revealed by the two-hybrid approach, were specific based on the use of B42/Ha-Ras protein as a negative control (Table 1). The precise amino acid residues within KSHV vBcl-2 involved in this multimerization are a matter for future investigations, but likely candidates are the three highly conserved domains present in many members of this multigene family[36].

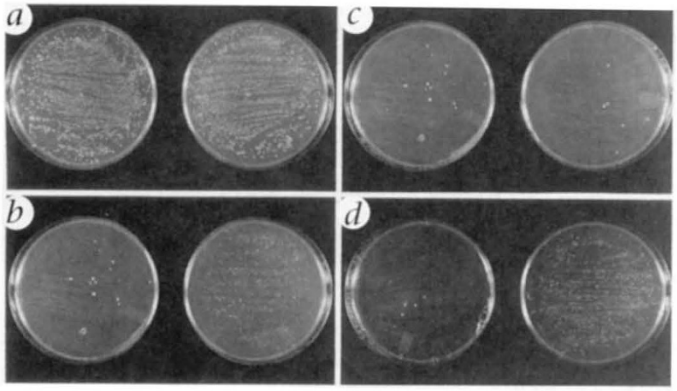

Fig. 4 KSHV vBcl-2 neutralizes Bax-mediated cytotoxicity in yeast. In each panel, the right hand plate contains galactose and the left hand plate contains glucose. *a*, Cotransfection of pEG-*bcl-2* with pJG4-5-KSHV *vbcl-2* showed yeast growth on both plates. *b*, Cotransfection of pEG-*bax* with pJG4-5-KSHV *vbcl-2* showed growth only on galactose plate which induces expression of KSHV *vbcl-2*. *c*, Cotransfection of pEG-*bax* with pJG4-5-*lamin/c* showed no growth on either plate. *d*, Cotransfection of pEG-*bax* with pJG4-5-*bcl-2* showed yeast growth only on galactose plate which induces expression of human *bcl-2*.

Effect of *vbcl-2* on mammalian cell survival

The effect of KSHV *vbcl-2* on mammalian cell survival was examined using a transient transfection assay[37,38]. In this assay, plasmids expressing cell death and/or survival proteins were cotransfected into GM701 fibroblasts along with a plasmid expressing *E.coli* β-galactosidase. Twenty-four hours post-transfection, cells were fixed and stained with X-gal and blue staining cells were scored, by microscopic examination, as live (fibroblast-like) or dead (round and pyknotic). Transfection of human Bax in this assay resulted in a dramatic reduction in the number of blue cells compared to cells transfected with control vector plasmid or KSHV *vbcl-2* expression plasmid (Fig. 5), probably as a result of rapid cell death. In comparison, cotransfection of the human Bax-expressing plasmid with plasmid expressing KSHV *vbcl-2* into GM701 fibroblasts demonstrated a significant increase in the number of blue cells (live and dead), indicating that KSHV *vbcl-2* can partially reverse Bax induced cell death. No reversal of Bax toxicity was demonstrated when cotransfection was performed with a plasmid containing frame-shifted KSHV *vbcl-2* that codes for a 64 amino-acids translatable fusion product, indicating the specificity of the KSHV *vbcl-2* in preventing Bax mediated cell death (Fig. 5).

Localization of vBcl-2

GM701 cells were transfected with an epitope-tagged KSHV *vbcl-2* expression plasmid which allows detection of the recombinant protein with specific antibodies (pcDNA3.1/His, Invitrogen). Twenty-four hours following transfection immunofluorescence studies indicated that KSHV vBcl-2 can be detected intracellularly (Fig. 6). The specific punctate cytoplasmic and perinuclear distribution of the staining suggests that the protein is localized to organelle membranes as described for Bcl-2 (ref. 39).

Discussion

We have identified a viral protein which possesses a protective

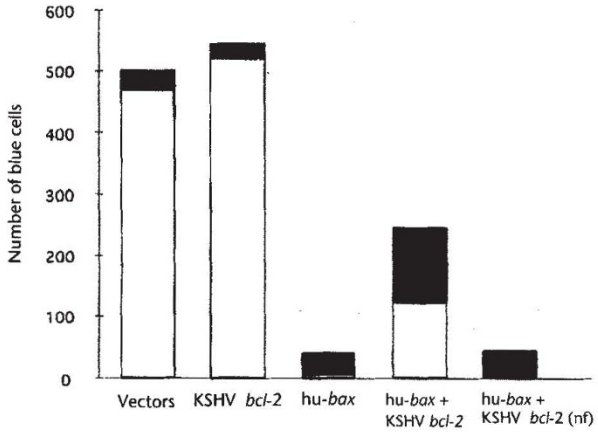

Fig. 5 Effect of KSHV v*bcl-2* on cell survival. GM701 cells transiently co-transfected with control pcDNA/His vector (0.7 μg) and reporter plasmid (0.2 μg) showed viable cells (open bar) and low numbers of dead cells (black bar). Cotransfection with KSHV v*bcl-2* (0.35μg), pcDNA/His vector (0.35 μg) and reporter plasmid (0.2μg) showed no deleterious effect on cell survival. Cotransfection of hu-*bax* (0.35 μg), pcDNA/His vector (0.35 μg) and reporter plasmid (0.2 μg) demonstrated low numbers of blue staining cells. Cotransfection of hu-*bax* (0.35 μg), KSHV v*bcl-2* (0.35 μg) and a reporter plasmid (0.2 μg) showed partial reversal of Bax toxicity. This reversal is not seen when KSHV v*bcl-2* was substituted with an out of frame expression construct, KSHV v*bcl-2*(nf). Data are expressed as the mean of live and dead cells counted from six determinations.

cell survival activity and interacts with other similar cell-survival regulatory proteins. Despite the difference in the predicted amino acid sequence of KSHV vBcl-2 compared to other Bcl-2 members it retains the structural characteristics necessary for the regulation of apoptosis and heterodimerization, a finding that can contribute to the ongoing mapping of functional domains in Bcl-2 and related proteins. Using RT-PCR we detected KSHV v*bcl-2* expression in a variety of KSHV-associated malignancies including classical (HIV seronegative) and AIDS-KS lesions from Italian and North American patients. Northern expression data indicate that this gene is expressed (confirmed by RT-PCR) at low levels under standard *in vitro* growth conditions in KSHV-infected BC-1 and is induced after treatment with the phorbol ester TPA. TPA is known to induce herpesvirus DNA replication, viral gene expression and virion production associated with lytic replication. This suggests that the primary pathogenic role of this gene product may be during the lytic viral phase of infection.

The function of KSHV v*bcl-2* in KSHV infection needs to be established. The detection of KSHV v*bcl-2* transcripts in KSHV-associated malignancies suggests a possible pathogenic role for this gene product analogous to *bcl-2*. The reciprocal antagonism between KSHV vBcl-2 and Bax appears to depend upon their ability to form heterodimers, giving rise to the hypothesis that the relative abundance of homo- or heterodimers of these proteins together with other apoptosis regulating proteins affect the cellular pathway. In this regard KSHV vBcl-2 can prevent early apoptotic cell death in virus-infected cells by changing the balance between these proteins to inhibit cell death and allow productive viral replication to occur. KSHV v*bcl-2* may even substitute for *bcl-2* in cells undergoing productive infection when virus induced shutdown of host cellular genes results in Bcl-2 deficiency. Additionally, KSHV encodes a unique, functional IL-6 homologue, KSHV ORFK2 which may also have anti-apoptotic and growth promoting effects in infected cells[40]. By promoting persistent KSHV infection and long-term survival of latently infected host cells, KSHV v*bcl-2* may co-operate with other cellular/viral oncoproteins to enhance cellular transformation[41].

No direct evidence has yet been published demonstrating that KSHV is an immortalizing and/or transforming virus. Nevertheless, KSHV is closely related to two other gammaherpesviruses (HVS and EBV) which have been shown to immortalize lymphocytes[42–44]. Both these viruses carry apoptosis inhibiting genes. Further, KSHV and HVS possess homologues to cellular D-type cyclins while EBV is able to induce transcription of cellular cyclin D2 (ref. 45–48). Expression of this spectrum of genes may contribute to a final common pathway for gammaherpesvirus-mediated cell transformation, relying in part on overexpression of cell cycle regulatory proteins[49].

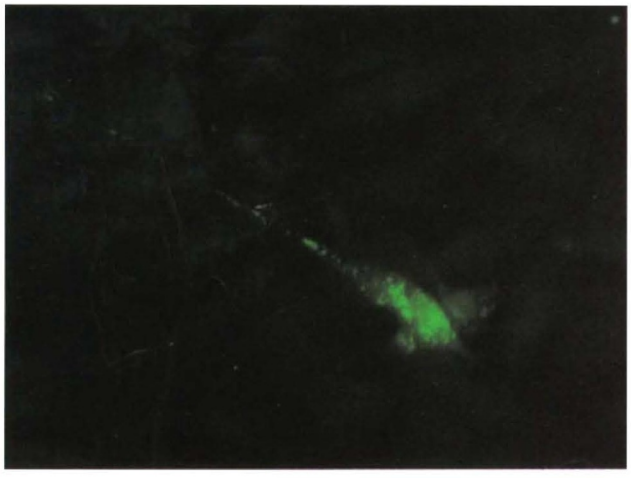

Fig. 6 Immunofluoresence localization of KSHV vBcl-2. GM701 fibroblasts cells were transfected with epitope-tagged KSHV v*bcl-2* expression plasmid. Punctate fluorescence reflects intracellular protein localization. Non-staining cells represent internal negative control completely devoid of fluorescence.

Methods

Reverse transcriptase PCR. Total RNA was isolated using RNAzol B (Tel-Test Inc., Texas) and contaminating genomic DNA eliminated with RNase free DNaseI (Promega, Madison, WI) followed by phenol-chloroform extraction. RT-PCR reactions were carried out on 1 μg RNA using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT) and a specific KSHV v*bcl-2* antisense primer 5'–TCCCCTTCTTCTGGTAAGCA–3'. The first strand cDNA samples were subjected to PCR using the same antisense primer and a sense primer 5'–ATGGACGAGGACGTTTTGCCT–3'. Amplification was for 35 cycles of 94 °C for 30 s and 60 °C for 60 s and expression detected using ethidium-bromide stained gels. Control reactions were performed with omission of the reverse transcriptase to exclude false-positive signals caused by DNA contamination. RT-PCR reactions were also performed using RNA extracted from an EBV infected cell line and in the absence of DNA. RT-PCR for the β-actin gene provided an RNA quality control.

Northern analysis. mRNA was extracted using a PolyATract mRNA isolation system (Promega, Madison, WI). 0.5 μg of RNA was separated on 1% agarose-formaldehyde gel and transferred to a nylon filter. The filter was probed with a riboprobe representing the anti-sense mRNA of KSHV v*bcl-2*

synthesized from a pBK-CMV plasmid (Stratagene, La Jolla, CA) template containing the KSHV *vbcl-2*.

Plasmids. Plasmids expressing the human Bcl-2, Bax, Lamin and Ras have been described[27]. The KSHV *vbcl-2* was subcloned in-frame into the two-hybrid plasmids, pEG202 and pJG4-5 (ref. 50) by PCR using primers containing *Eco*RI 5'–GGAATTCATGGACGAGGACGTTTTGCCT–3' and XhoI site 5'–CCCTCGAGTCATCCCCTTCTTCTGGTAAGCA–3' in the 5' and the 3' ends, respectively. Proper construction of plasmids and absence of PCR-generated errors were verified in every case by DNA sequence analysis. Sequences corresponding to the transmembrane domains were omitted and a stop codon was inserted to avoid problems with targeting of protein to the nucleus. The pEG202 plasmid uses an ADH promoter to constitutively drive expression of fusion proteins containing an N-terminal Lex-A DNA binding domain. The pJG4-5 plasmid utilizes a galactose-dependent promoter from the *GAL1* gene to inducibly drive expression of fusion proteins containing an N-terminal B42 trans-activation domain. The complete KSHV *vbcl-2* gene and the human *bax* genes were cloned into the expression vector pcDNA/His3.1 (Invitrogen, San Diego, CA) to generate mammalian expression plasmids with an epitope tag that allows detection of the recombinant protein with Anti-Xpress antibodies (Invitrogen, San Diego, CA). Frame-shifted KSHV *vbcl-2* was cloned in the same expression vector and encodes a 64 amino acid translatable fusion protein.

Yeast strains, media and transformation. *Sacchaaromyces cerevisiae* strains NA8711A and 9015 were grown in YPD broth medium (Bio 101, Inc, CA). Plasmid DNA transformations used 3 μg each of pEG202- and pGJ4-5-based expression plasmids according to the LiCl method[50]. Cells were grown in complete minimal medium lacking uracil, tryptophan or histidine as necessary to select for the presence of various plasmids. Plates were photographed at five days.

Yeast β-galactosidase assay. Yeast strain 9015 was stably transformed with *LexA* operator-lacZ reporter gene plasmid SH18-34 and selected for growth on uracil-deficient medium. Filter assays were performed using X-gal as a substrate, as described[27]. Filters were monitored for blue reaction products at 0.5, 1, 2, 4 and 24 h.

GM701 transient transfection assay. Human GM701 fibroblasts were maintained at 37 °C, 5% CO_2 in Modified Eagle Medium supplemented with 10% non-essential amino acids solution, 10% fetal calf serum, 4 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. Twenty-four h prior to transfection, cells were seeded into 24-well tissue culture plates at a density of 3.5×10^4 cells per well. Transfections were performed using the calcium-phosphate method employing the CellPhect Transfection Kit (Pharmacia Biotech, Uppsala, Sweden). At 24 h post transfection cells were washed twice with PBS, fixed at 4 °C with 0.2% gluteraldehyde, 2% paraformaldehyde, 49.2 mM sodium phosphate (pH 7.3) for 5 min and again washed twice with PBS. β-galactosidase expressing cells developed a blue color after staining cells with 80mM Na_2PO_4, 20 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 1 mg/ml X-gal, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ at 37 °C for 1–24 h. Blue cells were scored by microscopic examination as live or dead.

Immunofluorescence studies. GM701 cells were grown on chamber slides (Nunc, Inc. IL) and transfected with KSHV *vbcl-2* expression plasmid. Twenty-four h following transfection cells were washed with PBS and fixed for 60 min at 56 °C. The cells were blocked with 3% BSA in PBS (PBS-BSA) for 45 min and washed three times with 0.05% Tween-20 in PBS (PBS-Tween). Primary Anti-Xpress antibody (Invitrogen, San Diego, CA) was diluted 1:200 in PBS-BSA and incubated 30 min in a humid chamber followed by extensive washing with PBS-Tween. The secondary antibody, a goat antibody to mouse immunoglobilin coupled to fluorescein (Sigma), was diluted 1:100 in PBS-BSA and incubated for 45 min in a dark and humid chamber. The slides were then washed and mounted with Aqua-Mount (Lerner Laboratories, Pittsburgh, PA).

Acknowledgments

We thank Patrick S. Moore for the identification of the KSHV vbcl-2 sequences; Roger Brent, Erica A. Golemis and John C. Reed for the two-hybrid system and Riccardo Dalla-Favera for the CB33 cell line. This work was supported in part by NIH Grants CA67391 (Y.C and P.S.M.) and R01GM55147-01 (T.S.), a James S. McDonnell Foundation award (Y.C.), the American Cancer Society Institutional Research Grant IRG-177E (T.S.).

RECEIVED 16 JULY 1996; ACCEPTED 6 JANUARY 1997

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