Human cellular protein VRK2 interacts specifically with Epstein–Barr virus BHRF1, a homologue of Bcl-2, and enhances cell survival

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BHRF1, an early gene product of Epstein–Barr virus (EBV), is structurally and functionally homologous to Bcl-2, a cellular anti-apoptotic protein. BHRF1 has been shown to protect cells from apoptosis induced by numerous external stimuli. Nasopharyngeal carcinoma is an epithelial cancer associated closely with EBV infection. Specific proteins that might interact with and modulate the BHRF1 anti-apoptotic activity in normal epithelial cells are of interest. Therefore, a cDNA library derived from normal human foreskin keratinocytes was screened by the yeast two-hybrid system and a cellular gene encoding human vaccinia virus B1R kinase-related kinase 2 (VRK2) was isolated. Interaction between the cellular VRK2 and viral BHRF1 proteins was further demonstrated by glutathione S-transferase pull-down assays, confocal laser-scanning microscopy and co-immunoprecipitation. Analyses of VRK2-deletion mutants revealed that a 108 aa fragment at the C terminus was important for VRK2 to interact with BHRF1. For BHRF1, aa 1–18 and 89–142 were crucial in interacting with VRK2 and these two regions are counterparts of Bcl-2 homology domains 4 and 1. Overexpressed VRK2 alone showed a modest effect in anti-apoptosis and appeared to enhance cell survival in the presence of BHRF1. However, this enhancement was not observed when VRK2 was co-expressed with Bcl-2. The results indicate that human VRK2 interacts specifically with EBV BHRF1 and that the interaction is involved in protecting cells from apoptosis.

INTRODUCTION

In cell biology, apoptosis is involved in many important biological processes, such as normal development, tissue homeostasis, host defence and suppression of oncogenesis (Steller, 1995; Tsujimoto & Shimizu, 2000). It is a process of programmed cell death that is under tight genetic control. Severe diseases can result from aberrant apoptosis, e.g. malignancies, autoimmune and vascular diseases, AIDS and neurodegenerative disorders (Cohen, 1997; Nicholson, 1996; Thompson, 1995). In addition to many genes identified to be involved in the regulation of apoptosis, studies of the rapidly expanding Bcl-2 family have provided the most important insights into the control of apoptosis (Adams & Cory, 1998; Thompson, 1995; Vaux et al., 1994). Member proteins of the family can be cell-death agonists or antagonists and they share similarities in a transmembrane domain and four conserved Bcl-2 homology (BH) domains, BH1–BH4 (Antonsson & Martinou, 2000). These domains have been shown to mediate protein–protein interactions (Antonsson & Martinou, 2000; Borner et al., 1994; Farrow & Brown, 1996; Yin et al., 1994). Besides, there is mounting evidence for the association of Bcl-2 family members with other cellular proteins (Antonsson & Martinou, 2000; Lee et al., 1999; Liang et al., 1998; Reed, 1997; Xu & Reed, 1998; Zhang et al., 2000). Generally, the susceptibility of cells to diverse apoptotic stimuli is dictated by the relative ratio of anti-apoptotic and pro-apoptotic proteins (Oltvai & Korsmeyer, 1994). It is believed that protein–protein interactions between Bcl-2 family members or between...
Bcl-2 family members and other cellular partners are critical to the regulation of apoptosis (Yin et al., 1994).

Apoptosis is often induced when cells encounter virus infection and replication. Viruses have adopted various strategies to inhibit apoptosis of infected cells and some of them encode homologues of Bcl-2 (Hardwick, 1998). These will help viruses to establish and maintain persistent infections or to prolong survival of the infected cells and consequently maximize virus propagation (Everett & McFadden, 1999; Young et al., 1997). Therefore, anti-apoptotic effects contribute, at least in part, to the pathogenesis of virus infection and may promote the viral capability for oncogenesis (Young et al., 1997).

Epstein–Barr virus (EBV), a human gammaherpesvirus, is known to infect the vast majority of adults worldwide, usually with lifelong persistence (Rickinson & Kieff, 2001). It is an oncogenic virus that is implicated in the pathogenesis of a number of benign and malignant diseases. Apart from the well-known infectious mononucleosis, Burkitt’s lymphoma and nasopharyngeal carcinoma (NPC), infection with EBV also has been shown to be associated closely with other human illnesses, including lymphoproliferative diseases in immunocompromised patients, Hodgkin’s disease, T-cell lymphoma, gastric carcinoma and breast carcinoma (Bonnet et al., 1999; Brink et al., 2000; Oda et al., 1993; Pallesen et al., 1991; Shapiro et al., 1988; Su & Hsieh, 1992). The virus is able to infect B cells and stratified squamous epithelial cells and to transform them. EBV encodes a Bcl-2 homologue, BHRF1, that is expressed during its lytic cycle (Williams et al., 2001). Expression of the BHRF1 gene has been detected in several EBV-associated malignancies, such as B-cell lymphoma (Oudejans et al., 1995), NPC/T-cell lymphoma (Chiang et al., 1996), NPC (Liu et al., 2000) and, more recently, gastric carcinoma (Wang et al., 2004). The BHRF1 gene is highly conserved and is present in the genomes of all natural EBV isolates examined so far. Besides, the gene is also highly conserved in analogous viruses in other primates, indicating that BHRF1 has an evolutionarily conserved function (Williams et al., 2001). It shares limited sequence similarity with Bcl-2 (Heller et al., 1981; Khanim et al., 1997), with approximately 25 % amino acid sequence identity and 42 % amino acid sequence similarity (Marchini et al., 1991). Like Bcl-2, BHRF1 is located primarily in the mitochondrial, endoplasmic reticulum and nuclear membranes (Hickish et al., 1994; Khanim et al., 1997). The C terminus of BHRF1 contains a mitochondrial-targeting sequence and was suggested to affect cell physiology through interfering with mitochondrial function (Boya et al., 2004; D’Agostino et al., 2005; Kawanishi et al., 2002). Previous studies have demonstrated that BHRF1 protects various cell types from apoptosis following a broad range of external stimuli (Foghsgaard & Jäättelä, 1997; Henderson et al., 1993; Tarodi et al., 1994; Theodorakis et al., 1996). Thus, EBV BHRF1 is a structural and functional homologue of the cellular Bcl-2 protein. However, the three-dimensional structure of BHRF1 revealed important differences from that of Bcl-2, suggesting that the anti-apoptotic activity of BHRF1 may function through a different mechanism (Huang et al., 2003). As BHRF1 can repress apoptosis during EBV infection, the lifespan of the infected cells can be extended, enabling the virus to complete replication and dissemination. To explore the role of EBV BHRF1 in regulating cell apoptosis, we looked for cellular factors that can modulate the anti-apoptotic activity of BHRF1 through protein–protein interactions. Previously, we found that a cellular protein, PRA1, interacts specifically and selectively with and reduces the anti-apoptotic activity of BHRF1, but not Bcl-2 (Li et al., 2001). In this study, we investigated another cellular gene encoding the human vaccinia virus B1R kinase-related kinase 2 (VRK2) (Nezu et al., 1997) and described its effects on apoptosis, especially with BHRF1.

METHODS

Plasmid construction. Plasmids containing the full and deleted BHRF1 sequences of NPC origin (NBHRF1), bcl-2 and lamin were described elsewhere (Li et al., 2001). Briefly, the entire coding sequence of NBHRF1 was cloned in yeast expression plasmid pBTM116 and could express the LexA DNA-binding domain (LexA-DB)-tagged BHRF1. NBHRF1 cloned in Escherichia coli expression vector pGEX-5X-1 could express a glutathione S-transferase (GST)–BHRF1 fusion protein. Deletion mutants of NBHRF1, encoding aa 1–88, 1–142, 1–163, 19–191 and 61–191, were cloned in frame into pBTM116. For easy identification and constitutive expression of BHRF1 in mammalian cells, wild-type (WT) NBHRF1 and a deletion mutant encoding aa 19–191 were inserted into pcDNA3.1/NA-1 in frame with an EBV nuclear antigen 1 (EBNA-1) tag, which only encoded aa 408–446 of EBNA-1 and was located at the N terminus of the target protein. Plasmid pBTM/Bcl-2 contained the full-length human bcl-2 gene. Plasmid pBTM/lamin, containing a lamin gene, was used as a negative control.

Full-length VRK2 was amplified by PCR from a cDNA library of human normal foreskin keratinocytes (Clontech) and subsequently inserted into frame in pACT2 (Clontech), a vector that encodes the GAL4 activation domain (GAL4-AD). To construct VRK2-deletion mutants comprising aa 1–337, 94–337, 94–508, 175–508 and 401–508, all of the relevant DNA fragments generated by PCR were cloned in frame into pACT2. For transient expression of VRK2 in a mammalian cell system, a DNA fragment encoding the WT VRK2 was subcloned into vector pcDNA3-HA in order to generate an N-terminally haemagglutinin (HA)-tagged WT VRK2 protein. The nucleotide sequences of all of the recombinant constructs were confirmed by automated DNA sequencing.

Yeast two-hybrid screening. Yeast two-hybrid screening was used to pick up proteins interacting with the BHRF1 protein according to the method described by Vojtek & Hollenberg (1995). In brief, Saccharomyces cerevisiae strain L40 cells were co-transformed with the bait plasmid pBTM/NBHRF1 and the cDNA library of human normal foreskin keratinocytes, in which the cDNAs were fused to the GAL4-AD domain. Interacting clones were then selected to grow on plates with synthetic complete medium lacking Trp, Ura, Leu, Lys and His (SC-Trp, Ura, Leu, Lys, His) and SC-Trp, Ura, Leu, Lys as a control. Colonies that could grow on SC-Trp, Ura, Leu, Lys, His plates were able to produce His and were further tested for β-galactosidase activity 3 days later by a colony-lift filter assay (Vojtek & Hollenberg, 1995). Interaction between BHRF1 and any
proteins encoded by the cDNA library will activate the HIS3 and lacZ reporter genes. Through sequential purification and curing processes, cDNAs from the positive clones were prepared and their sequences were determined by using an ABI Prism Dye Terminator cycle sequencing core kit and an ABI 373A DNA sequencer (Perkin-Elmer). Obtained DNA sequences were then subjected to a BLAST search of the GenBank database.

**Cell lines and transfection.** A human embryonic kidney cell line, 293 (Graham et al., 1977), and RHEK-1 epithelial cells (Rhim, 1989) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum plus 100 units penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. N27-2, a stably transfected RHEK-1 cell line characterized by doxycycline (Dox; a tetracycline derivative)-inducible BHRF1 expression, and cell line V10-3, a vector-transfected control clone, were adopted (Li et al., 2001) and maintained in medium containing 400 μg neomycin ml⁻¹ (Gibco-BRL). Transfections were carried out by using a modified calcium phosphate method as described previously (Li et al., 2001) and transfection efficiencies were around 70–80%.

**Immunofluorescence assay.** Immunofluorescence staining was performed as described previously (Li et al., 2001). Briefly, N27-2 cells were grown on glass slides and transfected with HA-tagged VRK2. The cells were then induced with 1 μg Dox ml⁻¹ for 24 h, fixed with acetone at room temperature for 10 min and then probed with mouse anti-HA mAb (BAbCO) and rabbit anti-BHRF1 polyclonal antisera as primary antibodies for 1 h at 37 °C. Secondary antibodies were then applied to detect the primary antibodies, using Rhodamine red-X-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (both from Jackson ImmunoResearch). Stained cells were viewed by using a confocal laser-scanning microscope (Axiovert 135M photomicroscope; Zeiss).

**Western blotting and co-immunoprecipitation assays.** Proteins were extracted from mammalian cells in lysis buffer [20 mM octyl glucoside, 0-5% Triton X-100, 0·3 M NaCl, 25 mM sodium phosphate (pH 7·4), 10 μg leupeptin ml⁻¹, 10 μg aprotinin ml⁻¹ and 1 mM PMSF]. Extraction of proteins from yeast cells was carried out following the method described by Sato et al. (1994). The extracts were boiled at 100 °C for 5 min to denature the proteins and subjected to separation by SDS-PAGE. The separated proteins were blotted onto Hybond-C Super nitrocellulose membrane (Amersham Biosciences) by electrophoresis and reacted with specific antibodies. Specific antibodies used in the probing reactions were rabbit anti-GAL4-AD (Clontech), anti-GST (generated in vitro by expression of the glutathione-S-transferase gene in E. coli), mouse mAbs including anti-GAL4-AD (Clontech), anti-GST (generated in vitro by expression of the glutathione-S-transferase gene in E. coli) and anti-His (clone AC-15; Sigma).

In the co-immunoprecipitation assays for HA-tagged VRK2 and BHRF1, extracts from cells transfected with properly expressing plasmids were incubated with anti-HA or anti-BHRF1 3E8 antibody at 4 °C overnight. The immunocomplexes were then adsorbed by protein A-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C, washed thoroughly with Tris-buffered saline and finally subjected to Western blot analysis.

**GST pull-down assay.** Purified GST–BHRF1 fusion protein and GST control were bound to glutathione–agarose slurry in binding buffer [25 mM Tris/HCl (pH 7·5) and 125 mM NaCl] for 4 h at 4 °C and incubated with in vitro transcription and translation lysates of the [³⁵S]methionine-labelled HA-tagged VRK2, which had been produced by using a TNT coupled reticulocyte lysate system (Promega) in the presence of [³⁵S]methionine (Perkin-Elmer). The GST–protein complexes were then eluted from the agaroose beads by 2× SDS sample buffer [125 mM Tris/HCl (pH 6·8), 20% glycerol, 4% SDS, 3% dithiothreitol, 0·01 mg bromophenol blue ml⁻¹] and subjected to SDS-PAGE. [³⁵S]Methionine-labelled HA-tagged VRK2 was visualized by a phosphorimag (Storm 840; Molecular Dynamics).

**Assessment of cell growth by WST-1 assay.** BHRF1-inducible N27-2 cells and 293 cells were transfected transiently with tested plasmids. After 24 h, the transfected N27-2 cells were reacted with 1 μg Dox ml⁻¹ for 1 day to induce expression of BHRF1 and then the cells were trypsinized and seeded in 96-well plates at 4000 cells per well. Following 1 day incubation, apoptosis was induced by treating the cells with 0, 50 or 100 μM cisplatin for 72 h. For 293 transfectants, cells were plated at 6000 cells per well in 96-well plates and treated with 0, 40 or 80 μM cisplatin for 24 h. Viabilities of the tested cells were determined by a WST-1 assay in which cell-proliferation reagent WST-1 (Roche Molecular Biochemicals) was applied as described by the manufacturer. Student’s t-test was used to evaluate the effects of VRK2 on the anti-apoptotic activities of BHRF1 and Bcl-2. Compared experiments were regarded as significantly different when probability (P) values were <0·05.

**RESULTS**

**VRK2 interacts specifically with BHRF1**

Previously, we carried out yeast two-hybrid screening of a cDNA library from human normal foreskin keratinocytes using full-length BHRF1 as bait (Li et al., 2001). Several clones were identified to interact with BHRF1 specifically and reproducibly. One of them contains cDNA sequence corresponding to the coding sequence of the VRK2 protein (Neyz et al., 1997).

To corroborate the specificity of the BHRF1–VRK2 interaction, we cloned the full-length cDNA sequence of VRK2 into pACT2 and transformed yeast L40 cells together with the bait construct expressing LexA-DB-tagged BHRF1. Co-expression of the recombinant BHRF1 and VRK2 fusion proteins was demonstrated by Western blotting using specific antibodies, as shown in Fig. 1(a, b). The two-hybrid interaction assays showed that co-transformation of cells with BHRF1 and VRK2 resulted in activation of the HIS3 and lacZ reporter genes, as indicated by growth of the transformants on an SC-Trp, Ura, Leu, Lys, His plate (a His⁻ plate) and the formation of blue colonies on the colony-lift filter with a β-galactosidase assay. In control experiments, BHRF1 alone or co-expression of VRK2 and lamin failed to activate the HIS3 and lacZ reporter genes (Fig. 1c). These results indicate that VRK2 interacts specifically with BHRF1. To verify the interaction between BHRF1 and VRK2 in vitro, a GST pull-down assay was performed. Fig. 2(a) is reproduced with permission from our previous work (Li et al., 2001) and is shown here to demonstrate the protein products of purified GST and GST–BHRF1 fusion proteins. Fig. 2(b) exhibits in vitro-translated, [³⁵S]Methionine-labelled, HA-tagged VRK2. The [³⁵S]Methionine-labelled, HA-tagged VRK2 could be pulled down specifically by immobilized GST–BHRF1, but not by GST alone (Fig. 2c).

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To determine whether VRK2 and BHRF1 co-localize in cells, BHRF1-inducible stable cell clone N27-2 was transfected transiently with HA-tagged VRK2 and treated with Dox to induce BHRF1 expression. These two proteins were detected by reacting with mouse anti-HA mAb and rabbit anti-BHRF1 antiserum and their distributions were located by confocal laser-scanning microscopy. As shown in Fig. 3, HA-tagged VRK2 appeared to have a pattern of cytoplasmic staining in the transfected cells that was virtually identical to that of BHRF1. These observations indicate that VRK2 co-localizes with BHRF1 in cells, suggesting that the two proteins may interact in vivo. To confirm that BHRF1 interacts with VRK2 in vivo, immunoprecipitation experiments were carried out. The HA-tagged VRK2 expression construct or control plasmid pcDNA3-HA was transfected transiently into the BHRF1-inducible stable clone N27-2 and a vector-containing clone, V10-3 (data not shown), separately. The transfected cells were then incubated with or without 1 μg Dox ml⁻¹ for 24 h. Lysates of the transfectants were subjected to immunoprecipitation with monoclonal anti-HA or anti-BHRF1 antibody. Expressions of the target proteins were detected by Western blotting with specific antibodies as illustrated in Fig. 4(a). Both of the recombinant proteins were clearly expressed in the N27-2 cells. From the results shown in Fig. 4(b), BHRF1 could be precipitated from HA-tagged VRK2-transfected N27-2 cells by using anti-HA antibody. The interaction was also observed in a reciprocal experiment (data not shown). These results support the hypothesis that BHRF1 does interact with VRK2 in mammalian cells.

Fig. 1. Interaction of BHRF1 with VRK2 in the yeast two-hybrid system. (a) Saccharomyces cerevisiae strain L40 transformed with pBTM116 vector expressing the LexA DNA-binding domain (LexA-DB) or a plasmid expressing LexA-DB-tagged BHRF1 fusion protein (BHRF1) was analysed by Western blotting using anti-LexA-DB antibody (top) or anti-BHRF1 antibody (bottom). (b) Cell lysates containing pACT2 vector expressing the GAL4 activation domain (GAL4-AD) or a plasmid expressing GAL4-AD-tagged VRK2 fusion protein (VRK2) were analysed by using anti-GAL4-AD antibody. Molecular mass markers are indicated in kDa. (c) The interaction between BHRF1 and VRK2 was represented by the activation of the HIS3 and lacZ reporters. Transformants co-expressing BHRF1 or lamin with VRK2 or GAL4-AD were tested by growing on an SC-Trp, Ura, Leu, Lys plate (His⁺ plate) (left) and an SC-Trp, Ura, Leu, Lys, His plate (His⁻ plate) (middle). A colony from the His⁻ plate was tested for β-galactosidase activity (β-Gal) (right) by using a colony-lift filter assay, as described in Methods.

Fig. 2. Interaction of BHRF1 with VRK2 in GST pull-down assays. (a) Purified GST and GST–BHRF1 fusion proteins were detected by Western blotting using anti-GST antibody (top) and anti-BHRF1 antibody (bottom). Reproduced with permission from Li et al. (2001). (b) In vitro-translated, [35S]methionine-labelled and N-terminally HA-tagged VRK2 protein was visualized after SDS-PAGE with phosphorimaging. –, In vitro translation without DNA template served as a negative control. (c) The [35S]-labelled and N-terminally HA-tagged VRK2 was incubated separately with GST and GST–BHRF1 fusion proteins immobilized on glutathione–agarose. After extensive washing, labelled proteins were eluted from the agarose beads and analysed by SDS-PAGE and phosphorimaging. Molecular mass markers are indicated in kDa.

Fig. 3. Co-localization of BHRF1 and HA-tagged VRK2. A BHRF1-inducible stable clone, N27-2, was transfected transiently with N-terminally HA-tagged VRK2 and treated with Dox to induce BHRF1 expression. The cells were co-incubated firstly with polyclonal rabbit anti-BHRF1 serum and monoclonal mouse anti-HA antibody and secondly with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (a) and Rhodamine red-X-conjugated goat anti-mouse antibody (b). Finally, the cells were examined by using confocal laser-scanning microscopy. (c) Merged image of (a) and (b).
Identification of binding regions in VRK2 and BHRF1

To delineate the regions of VRK2 required for the interaction with BHRF1, a series of deletion mutants of VRK2 was generated and examined for binding abilities to BHRF1 by two-hybrid assays. Expression of the deletion mutants was demonstrated by Western blotting using anti-GAL4-AD antibody (Fig. 5a). Results of the two-hybrid assays showed that WT VRK2, truncations VRK2-(175–508) and VRK2-(401–508) could interact with BHRF1, as demonstrated by growth of colonies on the His\(^2\) plate (Fig. 5c) and production of blue colour on the colony-lift filter by \(\beta\)-galactosidase assay (Fig. 5d). Mutants VRK2-(1–337) and VRK2-(94–337) displayed no interaction with BHRF1 (Fig. 5b, c), suggesting that the last 108 residues at the C terminus of VRK2 are important for the interaction. However, mutant VRK2-(94–508), although including the last 108 aa, showed no reaction with BHRF1 (Fig. 5b, c). The interaction may require a particular conformation of VRK2, and this specialized structure could be lost due to the deletion of the first 93 aa. Compared with the positive reaction of the mutant VRK2-(175–508), another possibility cannot be excluded, i.e. that an inhibitory/ regulatory domain may reside in aa 94–174. Similar results were also obtained with HA-tagged VRK2 truncation mutants by co-immunoprecipitation assays in which N27-2 cells were transfected transiently with each of the mutants, separately (data not shown). To dissect the regions of BHRF1 involved in binding VRK2, various deletion fragments of BHRF1 were cloned into pBTM116 as described elsewhere (Li et al., 2001) and abilities of the mutants to interact with VRK2 were analysed by using the same method. Expression of the WT BHRF1 and its deletion mutants was demonstrated previously on a Western blot in this laboratory (Li et al., 2001). Only the results of two-hybrid interaction are shown in Fig. 5(e–h). The WT BHRF1 and mutants BHRF1-(1–163) and BHRF1-(1–142), which were truncated by 28 and 49 aa at the C terminus, respectively, were capable of interacting with VRK2. However, BHRF1-(19–191), BHRF1-(61–191) and BHRF1-(1–88) were unable to bind VRK2. This suggests that the last 49 aa at the C terminus of BHRF1, containing the BH2 and transmembrane domains, were not required for the interaction with VRK2. On the other hand, the first 18 aa (including the BH4 domain) and aa 89–142 (including the BH1 domain) of BHRF1 appeared important for binding VRK2.

Co-expression of VRK2 and BHRF1 enhances cell survival

The effect of VRK2 on the anti-apoptotic activity of BHRF1 was investigated by co-expressing these two proteins in the same cells. 293 cells were co-transfected transiently with the plasmid expressing EBNA-1-tagged WT BHRF1 and increasing amounts of the plasmid expressing HA-tagged WT VRK2. The transfected cells were challenged with a DNA-damaging agent, cisplatin, and assessed for cell viability by using the WST-1 assay. The percentage of viable cells was calculated and compared with that of vector-transfected cells without cisplatin treatment. Protein-expression levels of each set of transfectants were monitored by Western blotting (Fig. 6a). Surprisingly, only WT VRK2 alone slightly improved the resistance of cells to cisplatin-induced death and the effect was in proportion to the increasing concentrations of the plasmid for transfection (Fig. 6b, odd lanes). It suggests that VRK2 itself displayed some anti-apoptotic activity, although this was obviously weaker than that of BHRF1. Co-transfection of both VRK2 and BHRF1 resulted in higher cell viability (Fig. 6b, even lanes) and the anti-apoptotic effect became more and more profound when VRK2 levels were increased.
VRK2 interacts weakly with and imparts no significant effect on anti-apoptotic activity of Bcl-2

To understand whether Bcl-2, the cellular counterpart of BHRF1, also interacts with VRK2, the open reading frame of Bcl-2 was cloned into pBTM116 and its ability to bind GAL4-AD-tagged VRK2 was examined by yeast two-hybrid assays (Table 1). The results showed that the lacZ reporter gene was activated to produce blue colonies within 30 min when BHRF1 and VRK2 were co-expressed, whereas formation of faint blue colonies was not observed within the first 8 h when BHRF1 was replaced by Bcl-2. This weak reaction was not due to low-level expression of Bcl-2 protein and, in fact, it was expressed at a higher level than BHRF1 (data not shown). In control, no appreciable interaction was detected in cells with co-expression of VRK2 and lamin. It indicates that VRK2 interacts only weakly with Bcl-2 in a much less efficient way than its interaction with BHRF1.

In addition, we wished to elucidate whether VRK2 also has the same impact on the anti-apoptotic activity of Bcl-2. 293 cells were transfected transiently with HA-tagged WT VRK2 in combination with Bcl-2, EBNA-1-tagged WT BHRF1 and EBNA-1-tagged BHRF1-(19–191). Expression of target proteins in the transfectants was analysed by Western blotting (Fig. 7a). The transfected cells were treated with cisplatin to induce apoptosis and then assessed for cell viability by WST-1 assay. Fig. 7(b) shows that the lowest viability was obtained when cells were transfected by vector control, and it increased with WT VRK2 and more with WT BHRF1 (Fig. 7b, lanes 1–3 and 9–11). As shown previously, the WT VRK2 and the WT BHRF1 together produced a higher survival rate than either expressed alone (Fig. 7b, lanes 4 and 12). Although the mutant BHRF1-(19–191) is defective in binding VRK2, it still retained an anti-apoptotic function similar to that of WT BHRF1 (Fig. 7b, lanes 5 and 6).
was not improved significantly when Bcl-2, unlike BHRF1, was co-expressed with VRK2 (Fig. 7b, lanes 8 and 16). The enhancement effect on anti-apoptosis apparently does not exist between Bcl-2 and VRK2.

### DISCUSSION

The VRK2 protein was first identified by Nezu et al. (1997) as a novel human putative serine/threonine protein kinase and was discovered to share approximately 40% amino acid identity with a vaccinia virus serine/threonine protein kinase, B1R. These two genes were thought to be closely related members of a new protein kinase family. They also found that expression of VRK2 was widespread and elevated in highly proliferative tissues, such as fetal liver, testis and thymus and several cancer cell lines. The B1R kinase was reported to be expressed early during viral infection and to play an essential role in viral DNA replication (Condit & Motyczka, 1981; Condit et al., 1983; Kovacs et al., 2001; Rempel et al., 1990; Traktman et al., 1989). Based on the structural similarity between VRK2 and vaccinia virus B1R kinase, VRK2 was predicted to be involved in cell-growth regulation, including control of the cell cycle, DNA replication and transcription of certain genes. VRK2 also was suggested to play a role in germ-cell development (Lu & Bishop, 2003) and embryonic development of haematopoiesis in harmony with VRK1 and VRK3, two other members of the same new gene family (Vega et al., 2003). VRK2 has a longer C terminus [residues 397–508, including the last 108 aa (BHRF1-binding domain)] than its homologues VRK1 and B1R. A BLAST search of this fragment revealed no significant similarity to other known proteins and no obvious protein-interaction motif except for an extensive hydrophobic domain around aa 492–508. VRK2 shows modest autophosphorylation activity and can phosphorylate casein. Its highly hydrophobic C terminus makes the protein appear to be membrane-associated and localize at the endoplasmic reticulum (Nichols & Traktman, 2004). Recently, the kinase was reported to be involved in maintaining nuclear architecture by interacting with the BAF protein (Nichols et al., 2006). The exact physiological function of VRK2 is still not quite clear and requires more investigation to explore its biological roles. In this study, we demonstrated that VRK2 interacts specifically with EBV BHRF1 both in vitro and in vivo. Functional studies showed that VRK2 itself possessed a modest anti-apoptotic activity and co-expression with BHRF1 increased the survival rate of cells. However, this interaction could barely be detected between VRK2 and Bcl-2. Structurally, BHRF1 and Bcl-2 are different by three-dimensional analysis (Huang et al., 2003). Functionally, certain differences have been reported, i.e. that the proliferation rate of BHRF1-expressing SCC12F cells was higher than that of Bcl-2-expressing SCC12F cells and that BHRF1, but not Bcl-2, facilitated rapid transit through the cell cycle (Dawson et al., 1998). The specific interaction with VRK2 that we detected provides another special character to differentiate BHRF1 from Bcl-2.
Mapping the regions of BHRF1 responsible for its interaction with VRK2 showed that the first 18 aa (including the BH4 domain) and aa 89–142 (including the BH1 domain) are critical. Mutant BHRF1-(19–191) is deleted of the first 18 aa and is not able to bind VRK2, but still retains the ability to protect cells from apoptosis. Intact VRK2 showed a modest anti-apoptotic effect; however, it was not obvious in the co-transfection experiment with BHRF1-(19–191). A hypothesis is proposed that VRK2 and BHRF1 interact with the same target in order to display their anti-apoptotic activities. They compete for the same binding site on the target, but VRK2 has lower affinity. If both proteins are present, BHRF1 will have the privilege to exert its function. However, VRK2 can still function on the target indirectly by binding BHRF1, which may serve as a bridge. If VRK2 cannot bind BHRF1, for instance in the presence of BHRF1-(19–191), its modest anti-apoptotic effect cannot be shown. The reasons are that VRK2 cannot work on the assumed target through direct or indirect binding due to its lower affinity than that of BHRF1-(19–191) and inability to bind BHRF1-(19–191). The hypothesis may also apply to the relationship between Bcl-2 and VRK2. It can explain why the anti-apoptotic effects did not make much difference when Bcl-2 was expressed in the presence or absence of VRK2.

Of note, we demonstrated previously that the cellular protein PRA1 interacts with BHRF1, but not with Bcl-2, and reduces the anti-apoptotic activity of BHRF1 (Li et al., 2001). Coincidentally, the PRA1-interacting domains on BHRF1 co-localize with the VRK2-interacting domains and, therefore, it is very possible that PRA1 and VRK2 compete for the same sites to bind BHRF1. By selective interaction with these two cellular proteins, BHRF1 may play some roles in controlling the fate of cells to die or survive and, subsequently, regulate cell growth. Besides, there are many other host-specific apoptosis-modulatory proteins that are reported to interact with BHRF1, such as R-ras, Raf-1, Bak, Bik, Nip1-3 and HAX-1 (Matsuda et al., 2003; Theodorakis et al., 1996). The delicate and complicated balance of protein–protein interactions may decide between cell survival or death and may coordinate regulatory mechanisms, thereby to influence the viral life cycle and determine the extent of virus infection.

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</tr>
<tr>
<td>Bcl-2</td>
<td>BHRF1</td>
</tr>
<tr>
<td>Lamin</td>
<td>Blank</td>
</tr>
</tbody>
</table>

**Table 1.** Analysis of the interaction between VRK2 and BHRF1 or Bcl-2 by using yeast two-hybrid assays

ND, Not done; + +, positive reaction within 30 min; +, positive reaction after 8 h; −, lack of positive reaction after 24 h.

**Fig. 7.** Comparison of effects of VRK2 on the anti-apoptotic activities of BHRF1 and Bcl-2. (a) 293 cells were co-transfected transiently with N-terminally HA-tagged WT VRK2 and expressing construct of Bcl-2, N-terminally EBNA-1-tagged WT BHRF1 or BHRF1-(19–191), respectively. Cells co-transfected with VRK2 and vector were taken as the control. Expression of target proteins was analysed by Western blotting with anti-HA, anti-EBNA-1, anti-Bcl-2 and anti-β-actin antibodies. (b) The transfected cells were treated with cisplatin at the indicated concentrations for 24 h and then assessed for cell viability by WST-1 assay. The percentage of viable cells was calculated relative to the vector-transfected cells without cisplatin treatment. Data are presented as the mean ± SD of three separate experiments performed in triplicate. Difference between two sets of experiments was analysed by χ² test. When P<0.05, the difference was regarded as significant and marked by *. Molecular mass markers are indicated in kDa.

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