Distinct Roles of Kaposi’s Sarcoma-Associated Herpesvirus-Encoded Viral Interferon Regulatory Factors in Inflammatory Response and Cancer

Petra Baresova, Paula M. Pitha, Barbora Lubyova

Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiologic agent associated with Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman disease (MCD). Similar to other herpesviruses, KSHV has two life cycles, latency and lytic replication. In latency, the KSHV genome persists as a circular episome in the nucleus of the host cell and only a few viral genes are expressed. In this review, we focus on oncogenic, antiapoptotic, and immunomodulating properties of KSHV-encoded homologues of cellular interferon regulatory factors (IRFs)—viral IRF1 (vIRF1) to vIRF4—and their possible role in the KSHV-mediated antiviral response, apoptosis, and oncogenicity.
a certain degree of protein homology to cellular IRFs (Fig. 1B) (32); however, they do not contain five tryptophan residues in the DNA-binding region and are not able to bind to the promoters of type I IFN or ISGs.

### vIRF1 (ORF K9)

**Transcriptional regulation.** vIRF1 (449 amino acids) is a lytic gene (10, 33), expression of which can be induced by treatment with 12-0-tetradecanoyl-phorbol-13-acetate (TPA) of KSHV-positive primary effusion lymphoma (PEL) cells (14, 34, 35). However, low levels of vIRF1 were detected in latently infected KS and PEL cells, where it colocalizes to promyelocytic leukemia (PML) bodies (35, 36). The vIRF1 protein has a short half-life and does not remain at high levels throughout the lytic cascade (35). Transcription from the vIRF1 promoter can be upregulated by the KSHV-encoded replication and transcription activator (RTA) (37). However, the direct interaction of RTA with the vIRF1 promoter region has not been observed (38). Thus, it is likely that RTA transactivates the vIRF1 promoter through interaction with other DNA-binding factors.

**Protein structure and DNA-binding properties of vIRF1.** The vIRF1 protein is comprised of 449 amino acids. Out of all four

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**FIG 1** Homology and gene arrangement of KSHV-encoded vIRFs. (A) Gene arrangement of the vIRF locus in the KSHV genome. Open reading frames encoding vIRFs are located between ORF57 and ORF58 (83 to 95 kb). (B) Homology of vIRFs with cellular IRFs and rhesus macaque rhadinovirus (RRV)-encoded vIRFs. The degree of homology/identity was determined by BLASTp analysis using the UniProtKB database (http://uniprot.org).
KSHV vIRFs, vIRF1 has the highest degree of homology with cellular IRFs (Fig. 1B). The amino-terminal region of vIRF1 displays significant amino acid identity with the N-terminal DNA-binding domain (DBD) of IRF-9, IFN consensus sequence-binding protein (ICSBP/IRF-8), and IRF-4 (Fig. 1B) (32); however, the putative DBD of vIRF1 contains only two out of five conserved tryptophan residues. Using gel shift and chromatin immunoprecipitation assays, Park and colleagues (39) identified the vIRF1-binding consensus sequence located in the promoter region of K3 (viral E3 ubiquitin ligase), viral dihydrofolate reductase (vDHFR; ORF2) and viral IL-6 (vIL6). Consistent with this finding, vIRF1 overexpression resulted in the activation of K3:vDHFR: vIL6 promoter-reporter, and knockdown of vIRF1 expression in KSHV-positive BCBL-1 cells inhibited vIL6 transcription (39, 40), suggesting that vIRF1 may also play a role in the induction of certain KSHV genes. In agreement with these observations, recent studies of the crystal structure of the vIRF1 DBD revealed that vIRF1 is in fact a DNA-binding protein that might act directly on different operator sequences, as proposed for IRF-3 (41).

FIG 2 Schematic illustration of multiple immunomodulatory functions of KSHV within infected cells. Viral infection leads to activation of type I and II interferon signaling. Viral IRFs inhibit alpha, beta, and gamma interferon transcription, NF-κB activation, and MHC-I and II gene expression. P, phosphorus; TBK1, TANK-binding kinase 1.

vIRF1 AND IMMUNE RESPONSE
vIRF1 and interferon signaling. vIRF1 represses expression of interferon-inducible genes and blocks IRF-1- and IRF-3-mediated transcription (Fig. 2). Several reporter studies have shown that in a transient expression assay, vIRF1 has the ability to block the IFN-α/β-, IFN-γ-, or IRF-1-induced activation of interferon-stimulated gene (ISG) promoters, such as ISG-54 and ISG-15, gamma interferon-activated sequence (GAS)-element activation,
and IFN-β-induced p21WAF1/CIP1 transcription (33, 40, 42). Furthermore, vIRF1 efficiently inhibited Newcastle disease virus (NDV) activation of IFN-β promoter (10) and Sendai virus-induced expression of endogenous IFN-β, RANTES, and IP10 genes in human embryonic kidney (HEK293) cells (43).

The negative effect of vIRF1 on IFN-mediated signaling occurs through multiple protein-protein interactions between vIRF1 and cellular proteins. vIRF1 was reported to bind to IRF-1 and ICSBP/IRF-8 in vitro using a glutathione S-transferase (GST) pulldown assay (10). However, in another study, interaction between vIRF1 and IRF-1 could not be detected (33). Nevertheless, vIRF1 was shown to reduce both the DNA-binding affinity and transcription activity of IRF-1 (10, 33). Additionally, vIRF1 appears to interact with IRF-3 but does not affect IRF-3 dimerization, nuclear translocation, or DNA-binding activity (43).

In response to virus infection, cellular IRFs interact with and recruit the CBP/p300 transcriptional coactivator to type I IFN promoters. vIRF1 was shown to associate with the CBP/p300 co-activator and inhibit the formation of transcriptionally competent IRF-3-CBP/p300 complexes and consequently interfere with the recruitment of the basal transcription machinery (10, 43).

vIRF1 and MHC-I. vIRF1 is capable of downregulation of
basal and IFN-induced transcription of major histocompatibility complex class I (MHC-I) through its interaction with the transcriptional coactivator p300 (44). Thus, KSHV has evolved an efficient mechanism to evade antigen presentation, which results in the establishment of latency and host-pathogen equilibrium.

**vIRF1 AND CANCER**

vIRF1 and p53. vIRF1 function is not limited to the antiviral response. It also inhibits the function of p53 and modulates apoptotic signaling (Fig. 3). p53 is a transcription factor which responds to stress signals, such as DNA damage, or viral infections, by inducing cell cycle arrest or apoptosis (45). p53 activity can be regulated through specific posttranslational modifications, e.g., phosphorylation, acetylation, ubiquitination, and SUMOylation (46, 47). Upon DNA damage, p53 is phosphorylated by ataxia telangiectasia-mutated (ATM) kinase and acetylated by CBP/p300, which leads to p53 activation and protein stabilization (48). On the other hand, polyubiquitination of p53 by murine double minute 2 (MDM2) E3 ubiquitin-protein ligase targets p53 for 26S proteasome-mediated degradation (49). vIRF1 negatively regulates p53 function by engaging two mechanisms. (i) vIRF1 interacts with ATM and blocks its kinase activity that is induced by DNA damage stress signals. As a consequence, vIRF1 greatly reduces the levels of p53 phosphorylation on serine residue 15, resulting in an increase of p53 ubiquitination by MDM2, leading to p53 degradation (50, 51). (ii) vIRF1 directly interacts with the DNA-binding domain (DBD) of p53 and suppresses p53 acetylation, resulting in the inhibition of p53 activity and p53-mediated apoptosis (52). Expression of vIRF1 in Saos-2 (human osteosarcoma with null p53) cells significantly reduced p53-mediated apoptosis (reduction of apoptotic cells from 75% to 32%), with an increased accumulation of cells in the G2/M phase of the cell cycle (51).

**vIRF1 and TGF-β.** Inactivation of the transforming growth factor β (TGF-β) signaling pathway is important in the genesis of human malignancies (53, 54). Members of the TGF-β family regulate a variety of biological processes, including cell growth, differentiation, matrix production, and apoptosis (55, 56). TGF-β signaling results in the activation of members of the Smad family of tumor suppressors, which include Smad 2 and Smad 3. The activated Smads form complexes with a common mediator, Smad 4, and translocate to the nucleus, where they are involved in regulating the transcription of target genes (57, 58). TGF-β inhibits cell proliferation by regulating two classes of genes. First, TGF-β-activated Smads target the promoter of the c-myc gene, leading to transcriptional inhibition of c-Myc. Second, activated Smad complexes are involved in the induction of two cyclin-dependent kinase inhibitors, p15 and p21 (59–61). vIRF1 was shown to suppress the TGF-β/Smad signaling pathway (62). vIRF1 inhibited TGF-β–stimulated expression of a synthetic reporter containing four Smad elements. Direct interaction of vIRF1 with both Smad 3 and Smad 4 resulted in the inhibition of their transcriptional activity. In addition, vIRF1 interfered with Smad 3-Smad 4 complex formation and DNA binding (62).

**vIRF1 AND APOPTOSIS**

vIRF1 and the proapoptotic BOPs, Bim and Bid. Both Bim and Bid are induced during KSHV lytic replication and are very powerful negative regulators of viral replication (63). Bim and Bid, like other BH3-only proteins (BOPs), function by virtue of their BH3 domains to target antiapoptotic members of the Bcl-2 family and to disrupt their interactions with apoptotic executioner proteins Bax and Bak. This liberates Bax and Bak for oligomerization and mitochondrial permeabilization (64, 65). However, Bim and Bid can also interact with and activate Bax and Bak directly, via induced conformational changes (66, 67). Direct interaction of vIRF1 with proapoptotic proteins Bim (68) and Bid (69) leads to inhibition of cellular proapoptotic signals (Fig. 3). The vIRF1-mediated inhibition of Bim utilizes a unique mechanism of Bim regulation, via nuclear sequestration of Bim away from mitochondria (68). vIRF-1–mediated relocalization of Bim was identified in transfected cells by both an immunofluorescence assay and Western blot analysis of fractionated cell extracts. Coimmunoprecipitation assays and GST pulldown assays utilizing various vIRF1 deletion mutants identified the minimal region of vIRF1 (residues 174 to 181) sufficient for direct interaction with Bim. Blocking of the vIRF1-Bim interaction with a cell-permeable peptide corresponding to the Bim-binding region of vIRF1 led to reduced virus titers in KSHV-infected telomerase-immortalized endothelial (TIME) cells (68). Thus, vIRF1–induced nuclear localization and inactivation of Bim represents a novel mechanism of viral evasion from antiviral defenses of the host. Recently, the Bim-binding region (BBD) of vIRF1 was shown to interact also with the BH-3 domain of other BOPs, e.g., Bid, Bik, Bmf, Hrk, and Noxa (69). In contrast to Bim, vIRF1 was unable to mediate nuclear sequestration of Bid protein. Direct functional inhibition of Bid by vIRF1 was demonstrated by the ability of vIRF1 to block Bid-induced mitochondrial permeabilization in vitro (69). Furthermore, Western blot analysis of mitochondrial preparations from KSHV-positive BCBL-1 cells, or TIME cells, revealed mitochondrial association of endogenous vIRF1 in latent and lytic cultures. Thus, Bim and Bid are contributors to negative regulation of KSHV infection, and their targeting by vIRF1 may be important for productive virus replication (69).

**vIRF1 and GRIM19.** In PEL cells, vIRF1 was also shown to associate with the interferon/retinoid acid (IFN/RA)-inducible cell death regulator, GRIM19. GRIM19 encodes a 144-amino-acid protein that localizes predominantly to the nucleus and enhances caspase 9 activity and apoptotic cell death in response to IFN/RA (70). In the presence of vIRF1, GRIM19 was unable to induce apoptosis (71). The inhibition of apoptosis may be related to the ability of vIRF1 to induce cellular transformation. Overexpression of vIRF1 in NIH 3T3 cells resulted in the attenuation of growth regulation and contact inhibition. These cells were also able to grow as tumors in nude mice (42). However, coexpression of vIRF1 and GRIM19 in NIH 3T3 cells led to significant suppression of transformed colonies, suggesting that vIRF1–mediated inhibition of GRIM19 contributes to the cell-transforming properties of KSHV (72).

**vIRF1 and CD95L.** vIRF1 was shown to be able to inhibit activation-induced cell death (AICD) mediated by CD95 death receptor signaling. The CD95 (also called Fas/APO-1) pathway plays a major role in the induction of apoptosis in lymphoid and nonlymphoid tissues. CD95 (Fas/APO-1), a type I transmembrane protein, is a member of the tumor necrosis factor (TNF) receptor superfamily, which is expressed in various tissues (73, 74). The CD95 ligand (CD95L) is induced in response to a variety of signals, including IFN-γ and T cell receptor (TCR)/CD3 stimulation. CD95L induces apoptosis via formation of a death-inducing signaling complex and initiation of a signaling cascade of
vIRF2 (ORF K11 AND K11.1)

Transcriptional regulation. The initial studies carried out by Burysek et al. identified vIRF-2 as a constitutively expressed protein encoded by a single ORF (K11.1) consisting of 163 amino acids (77) (Fig. 1A). More recently, other groups have found that the vIRF-2 gene encodes a 2.2-kb spliced transcript representing two exons of ORFs K11.1 and K11, from which the full-length vIRF-2 protein is translated (680 amino acids). Moreover, this spliced form of vIRF-2 was characterized as an inducible gene from microarray and quantitative PCR studies (16, 78). vIRF2 is present in the cytoplasm and the nucleus of infected cells, and its expression can be induced by IFN treatment (79).

vIRF2 AND IMMUNE RESPONSE

vIRF2 and cellular IRFs. Studies with the short form of vIRF2 (K11.1; 20 kDa) demonstrated the binding of vIRF2 to a consensus NF-κB-binding site but not to an interferon-stimulated response element (ISRE) (77) (Fig. 2). Additionally, vIRF2 was shown to suppress IRF-1- and IRF-3-driven activation of an IFN-α reporter promoter in cells infected with NDV. In GST pull-down assays, this short form of vIRF2 interacted with cellular IRF-1, p300/CBP, p65, IRF-2, and ICSBP/IRF-8; however, it did not bind to IRF-3 (77).

Studies including full-length vIRF2 showed that vIRF2 downregulated both IFN-α- and IFN-λ-driven transactivation of reporter promoter containing ISRE (80). Furthermore, vIRF2 negatively regulated the transactivation of the ISRE promoter by IRF-1 as well as activation of IFN-β reporter promoter by either IRF-3 or IRF-1, but not by IRF-7 (80). Although interaction between the short form of vIRF2 and IRF-3 was not observed (77), full-length vIRF-2 was found to associate with IRF-3 (79). vIRF2 was shown to recruit caspase 3 to IRF-3 and thus accelerate the caspase-dependent process of IRF-3 turnover, leading to an inefficient antiviral response (79). Recently, it was also shown that vIRF2 inhibits type I IFN signaling by targeting components of the interferon-stimulated gene factor 3 (ISGF3) complex, STAT1 and IRF-9, which results in the inhibition of ISG expression (81). Thus, vIRF2 appears to be able to inhibit both early and later steps of the antiviral signaling pathway.

vIRF2 and PKR. The short form of vIRF2 (K11.1) also binds to the dsRNA-activated protein kinase (PKR) and blocks the auto-phosphorylation and phosphorylation of PKR substrates (82). PKR acts as a serine/threonine kinase that phosphorylates and activates downstream targets, including eukaryotic translation initiation factor 2 alpha (eIF-2α), histone 2A, and NF-κB, events that affect cell growth, cell differentiation, viral clearance, and induction of apoptosis (83–86). By inhibiting the kinase activity of PKR through vIRF2 and the consequent down-modulation of protein synthesis, KSHV has evolved a mechanism by which it can overcome the interferon-mediated antiviral effect (82). Thus, the anti-interferon functions of vIRF2 may contribute to the establishment of chronic or latent infection.

vIRF2 AND APOPTOSIS

vIRF2 and CD95L. Similarly to vIRF1, vIRF2 was able to inhibit CD95L expression (76) (Fig. 3). However, vIRF2 did not modulate binding of IRF-1 to CD95L promoter DNA. The possible mechanism of vIRF2-mediated repression of CD95L induction is through NF-κB inhibition (77). These data suggest that both vIRF1 and vIRF2 act as modulators of the immune system by repressing activation-induced cell death (AICD) via modulation of TCR/CD3-mediated induction of CD95L (87).

vIRF3 (ORF K10.5 AND K10.6)

Transcriptional regulation. vIRF3 (also called LANA2) is a spliced product of two ORFs, K10.5 and K10.6 (11) (Fig. 1A). vIRF3 (566 amino acids) is constitutively expressed in the nuclei of KSHV-infected hematopoietic tissues, including those from body cavity-based primary effusion lymphoma (PEL) and Castleman disease (CD), but not in Kaposi’s sarcoma (KS) lesions (88). Unlike other vIRFs, vIRF3 is not induced during lytic reactivation of TPA-treated PEL cells (89). vIRF3 shows a significant degree of homology with the IFN-association domain (IAD) region of cellular IRF-8/ICSBP (Fig. 1B)(11, 88, 90). IAD is a conserved region shared by all IRFs, excluding IRF-1 and IRF-2. IAD mediates homo- and heteromeric interactions that occur between IRFs or IRFs and other cellular factors, e.g., STAT1/2 and PU.1. (91).

vIRF3 AND IMMUNE RESPONSE

vIRF3 and cellular IRFs. Similarly to vIRF1 and vIRF2, vIRF3 is able to associate with activated nuclear IRF-3 and IRF-7 and consequently modulate the innate antiviral response by interfering with their functions (92) (Fig. 2). As shown recently, vIRF3 can also interact with IRF-5 (93, 94). The mapping of the vIRF3-binding domain revealed that vIRF3 associates with IRF-3 and IRF-7 through its C-terminal region (amino acids 254 to 566). Although vIRF3 is not a DNA-binding protein, it is recruited to IFN-α gene promoters via its interaction with IRF-3, IRF-7, and p300. We have shown that the presence of vIRF3 in the enhanceosome and its interaction with the IFN-α gene promoter increases the binding of IRF-3, IRF-7, and acetylated histone H3 to the promoters of IFN-α genes (90). In contrast, Joo et al. (92) showed that vIRF3 blocks cellular IRF-7-mediated innate immunity by interacting with the DNA-binding domain or the IRF-association domain of IRF-7. This interaction specifically suppresses IRF-7 DNA binding and consequently inhibits IFN-α-1, -α-4, and -α-6 gene expression, as estimated by real-time quantitative reverse transcription-PCR (qRT-PCR) in transfected HEK293T cells infected with Sendai virus (92). The vIRF3-mediated modulation of IRF-7 function may also be important in the context of Epstein-Barr virus (EBV) transformation, where IRF-7 is activated in EBV-transformed cells (95) and induces the expression of LMP-1 (96), which has a critical role in EBV-induced lymphomagenesis.

vIRF3 and IRF-5. Interferon regulatory factor 5 (IRF-5) is a transcription factor that has a key role in the induction of antiviral and inflammatory responses and autoimmunity (97, 98). It is induced by viral infection and type I IFN and activated by TLR7- and TLR9/MyD88-dependent pathways. Although IRF-5 is a direct
target of p53, its cell cycle regulatory and proapoptotic effects are p53 independent (27). IRF-5 is expressed in B cells, dendritic cells, and macrophages and remains in the cytoplasm as an inactive protein. Upon DNA damage signals or activation of the IFN, TLR7, or TLR9 pathways, IRF-5 is phosphorylated, K63 ubiquitinated, and subsequently translocated to the nucleus. In addition to its antiviral effect, its tumor suppressor properties and ability to regulate the cell cycle have also been shown (27, 99). Interestingly, in the nuclei of PEL cells, IRF-5 is present constitutively. The interaction of IRF-5 with vIRF3, which was demonstrated in transfected HEK293T cells and PEL cells, leads to the inhibition of IRF-5 binding to the promoters of IFN-β and interferon-responsive genes (ISGs) (94). Thus, vIRF3 blocks IRF-5-mediated activation of ISRE and IFN-β promoter reporter activity. In addition, vIRF3 antagonizes IRF-5-mediated activation of p21 promoter reporter and prevents IRF-5-mediated growth inhibition and G2/M cell cycle arrest (93, 94). Thus, the expression of vIRF3 in PEL cells may contribute to their tumorigenicity.

**vIRF3 and NF-κB.** Association with cellular IRFs is not the only mechanism by which vIRF3 downregulates the innate inflammatory response. Activation of NF-κB pathway is also part of the inflammatory response and can be induced by viral infection. NF-κB is a transcription factor that plays a role in innate and adaptive immune responses through its ability to regulate the production of cytokines, receptors necessary for immune recognition, and proteins participating in antigen presentation (100, 101). In unstimulated cells, NF-κB is localized in the cytoplasm in an inactive form, where it associates with the inhibitory protein IκB. Upon viral infection, IκB is phosphorylated, which leads to the ubiquitination and subsequent degradation of the IκB inhibitor. Phosphorylation of IκB is mediated by the IκB kinase (IKK) complex that is composed of three subunits: IKKα, IKKβ, and IKKγ. Liberated NF-κB can then translocate to the nucleus and activate transcription of target genes (102). Phosphorylation of IκB is thus the key step in regulation of NF-κB activation. The IKKβ subunit of IKK was shown to be targeted by vIRF3 (103). Direct interaction between vIRF3 and IKKβ, which was demonstrated in transfected HEK293T cells, led to hypophosphorylation of IκB, impaired translocation of IκB to the nucleus, and reduced NF-κB-mediated transcription (103). However, coimmunoprecipitation experiments failed to demonstrate IKKβ-vIRF3 interaction in KSHV-positive BCBL-1 cells (103). In agreement with previous reports demonstrating that suppression of NF-κB results in enhanced TNF-α-induced apoptosis, vIRF3 expression significantly increased TNF-α-induced apoptosis (estimated by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL]) in HEK293T cells (103).

**vIRF3 and MHC-II** Escape from antigen presentation is an effective strategy of KSHV, and vIRF3 plays an important part by interfering with the adaptive immune response by inhibition of major histocompatibility complex class II (MHC-II) and IFN-γ expression. MHC-II molecules are found on only a few specialized cell types, including macrophages, dendritic cells, and B cells, all of which are professional antigen-presenting cells (APCs). In humans, MHC-II expression is inducible by gamma interferon (IFN-γ) in almost every cell type (104). KSHV-positive PEL cells exhibit a lower level of MHC-II expression on the cell surface than that exhibited by KSHV-negative B cell lymphomas. It was shown that the small interfering RNA (siRNA)-mediated knockdown of vIRF3 in KSHV-infected PEL cell lines resulted in increased MHC-II levels (105). Conversely, overexpression of vIRF3 in KSHV-negative B cells (BJAB) led to down-modulation of MHC-II. The observed suppression of MHC-II is due to the vIRF3-mediated inhibition of the class II trans-activator (CIITA) which is a major regulator of MHC-II transcription. The expression of CIITA is under the control of the B cell type-specific promoter PIII or the IFN-γ-inducible promoter PIV. The presence of vIRF3 in PEL cells reduced the activity of PIII and PIV promoters. In addition, vIRF3 also downregulates IFN-γ promoter activity. A vIRF3 knockdown in PEL cells resulted in increased levels of IFN-γ protein (105). Suppression of IFN-γ signaling leads to down-modulation of MHC-II. Thus, inhibition of MHC-I and MHC-II by vIRF1 and vIRF3, respectively, contributes to viral immunoevasion and the effective escape from antigen presentation in KSHV-infected cells.

**vIRF3 AND CANCER** Oncogenesis is represented by multiple events which are characterized by the progression of cytological, genetic, and cellular changes that ultimately culminate in uncontrolled cell division and tumor growth. Oncogenesis requires inactivation of tumor suppressor genes and activation of cellular proto-oncogenes. In the case of virus-induced oncogenesis, attenuation of the antiviral response is also important. KSHV-encoded vIRF3 can promote tumorigenesis by at least two mechanisms: (i) association with a key tumor suppressor, p53, and downregulation of its transcription activity (88) and (ii) activation of the c-Myc proto-oncogene (106) (Fig. 3). vIRF3 is also involved in the regulation of apoptosis and the cell cycle and in the modulation of microtubule dynamics (107). Interestingly, vIRF3 is required for the survival of KSHV-infected PEL cells in vitro. Silencing of vIRF-3 by approximately 40% resulted in a 3-fold increase of caspase 3/7 activity and the subsequent induction of apoptosis (89). vIRF-3 can thus be considered a bona fide oncogene of KSHV.

**vIRF-3 and p53.** The p53 gene was the first identified tumor suppressor gene. Attenuation of p53 function is among the most frequent molecular events occurring in human cancers (108). vIRF3 was shown to interact with p53 protein in vitro. The interaction domain is located in the region consisting of amino acids 290 and 393 of p53. This region encompasses the tetramerization and regulatory domains of p53. Cells expressing vIRF3 show inhibition of p53-mediated apoptosis and lower levels of caspase 8 activation (88). However, the direct interaction between endogenous vIRF3 and p53 in KSHV-infected PEL cells has not been demonstrated, and thus the molecular mechanism by which vIRF3 modulates p53 function is yet to be determined.

**vIRF3 and c-Myc.** vIRF3 also stimulates the transcriptional activity of the c-Myc proto-oncogene. c-Myc is a transcription factor that activates expression of a majority of the transcriptionally active cellular genes (109) including those involved in the control of cellular growth, proliferation, and cell survival. Thus, c-Myc activation reduces growth regulation, drives cell proliferation, and blocks cellular differentiation. It also plays an important role in stem cell self-renewal (110, 111). Although c-Myc was originally thought to stimulate promoters of genes containing E-box consensus sequences (112), recent data indicate that c-Myc does not target a specific transcriptional program(s) but is a universal amplifier of gene expression, which increases the output of all active promoters (113). The E-box-dependent transcriptional activity of c-Myc can be repressed by Myc modulator 1 (MM-1)
and cell cycle regulation (117–119). In addition to PML, PODs are involved in gene transcription, genomic stability, apoptosis, domains, which form nuclear depots for a number of proteins that infection. It is a major component of PODs, also known as ND10 that has a role in tumor suppression and host defense against virus myelocytic leukemia (PML) protein is a multifunctional protein (PODs) and interaction with 14-3-3 regulatory proteins. The pro-

contribution to the disruption of PML oncogenic domains total transcriptional profile in PEL cells, as seen in other tumor cells (116).

vIRF3 and PODs. The multiple effects of vIRF3 on cellular transformation and carcinogenesis are strongly supported by its contribution to the disruption of PML oncogenic domains (PODs) and interaction with 14-3-3 regulatory proteins. The pro-

myelocytic leukemia (PML) protein is a multifunctional protein that has a role in tumor suppression and host defense against virus infection. It is a major component of PODs, also known as ND10 domains, which form nuclear depots for a number of proteins that are involved in gene transcription, genomic stability, apoptosis, and cell cycle regulation (117–119). In addition to PML, PODs contain other proteins, including Sp100, Daxx, pRb, CBP, and p53 (120). vIRF3 expression induces the displacement of PML or Sp100 from PODs and the degradation of PML by a proteasome-mediated mechanism (121). Moreover, silencing of vIRF3 expres-
sion in PEL cells by RNA interference led to an increase in the PML levels. vIRF3 also interferes with the PML-mediated transcrip-
tional repression of survivin, a protein that contributes to the malignant progression of PEL cells (121). Recently, vIRF3 was shown to covalently conjugate to small ubiquitin-like modifier 1 (SUMO1) and SUMO2 both in vitro and in latently KSHV-infected PEL cells (122). SUMO is an 11.5-kDa protein that has the ability to conjugate to multiple proteins and modulate protein stability, subcellular localization, and protein activity and function (123). It is believed that covalent conjugation of SUMO to vIRF3 may serve as a bridge between vIRF3 and other SUMO-interacting proteins that are required for vIRF3-mediated disruption of PODs (122). Importantly, vIRF3 was also shown to inhibit SUMOylation of three pocket proteins, pRb, p107, and p130, which are key tumor suppressors frequently targeted by oncoproteins expressed by DNA tumor viruses (124). vIRF3 contains an LXXCE motif that mediates the interaction with these cellular proteins and is required for inhibition of their conjugation to SUMO (124).

**vIRF3 and HIF-1.** Hypoxia-inducible factor 1 (HIF-1), a key regulator of cellular responses in low-oxygen concentrations, is involved in developmental and pathological angiogenesis. Under normal conditions, HIF-1α, an oxygen-sensitive subunit of HIF-1, undergoes rapid ubiquitination and proteasomal degrada-
tion (125). In contrast, under hypoxic conditions, HIF-1α is stabil-
ized, accumulated, and translocated into the nucleus, where it forms a heterodimeric complex with HIF-1β to activate transcrip-
tion of its target genes, including vascular endothelial growth fac-
tor (VEGF), which plays an important role in angiogenesis and tumor growth (126). By direct binding to HIF-1α, vIRF3 robustly induces HIF-1α transcriptional activity and its stability and thereby increases VEGF production, which may affect the prolif-
eration of neighboring cells by a paracrine mechanism and may facilitate endothelial tube formation (127). However, the expres-
sion of vIRF3 has not been detected in Kaposis’s sarcoma lesions; thus, its biological relevance may be limited to KSHV-positive B cells.

**vIRF3 and 14-3-3.** The 14-3-3 proteins are a family of highly conserved dimeric regulatory proteins which are involved in the regulation of the cell cycle, apoptosis, and oncogenesis (128). 14-

3-3 proteins promote the cytoplasmic localization of members of the forkhead box O (FOXO) family of transcription factors, FOXO1, FOXO3a, and FOXO4, resulting in the inhibition of their transcription activity (129). Activation of genes by FOXO members leads to G cell cycle arrest and apoptosis in many tumor cell lines (130, 131). Phosphorylation of FOXO3a by AKT generates binding sites for 14-3-3 proteins, which leads to translocation of FOXO3a from the nucleus to the cytoplasm, where it remains in an inactive form (132, 133). Phosphorylated vIRF-3 can interact with 14-3-3 proteins and FOXO3a. Importantly, vIRF3 is able to bind also to the nonphosphorylated form of FOXO3a. Thus, this interaction facilitates the binding between 14-3-3 and nonphosphorylated FOXO3a and inhibits the transactivation of FOXO3a targets, such as proapoptotic Bim. In addition, vIRF3 also blocks G/M cell cycle arrest, which is induced by 14-3-3 protein overexpression (134).

**vIRF3 AND APOPTOSIS**

**vIRF3 and PKR.** Activation of IFN-responsive genes (ISGs) is an important mechanism for the establishment of an effective anti-

viral immune response. vIRF3 was shown to target the function of IFN-induced dsRNA-activated protein kinase (PKR). The expres-

sion of vIRF3 in BSC-40 cells resulted in reduced levels of PKR-

mediated apoptosis as measured by DNA ladder formation (135). Furthermore, the presence of vIRF3 leads to decreased levels of phosphorylated elf-2α by PKR (135). Deregulation of the elf-2α checkpoint and consequent permissiveness to virus infection may be a common occurrence in tumorigenic mammalian cell lines (136). The direct interaction of vIRF-3 and PKR was not detected, and vIRF3 was not able to inhibit NF-kB activation in response to PKR. However, vIRF3 inhibited PKR-induced activation of caspase 3, but not that of caspase 9, suggesting that only the Fas-associated protein with death domain (FADD)/caspase 8 pathway is affected by vIRF3 (135).

Likewise, the work of several other groups has shown the associ-

ation of vIRF3 with the regulation of apoptosis. Rivas et al. showed that vIRF3 is able to inhibit p53- and doxorubicin-induced apoptosis in Saos-2 (human osteosarcoma with null p53) and U2OS (human osteosarcoma with wild-type p53) cells (88). In contrast to these findings, Seo et al. reported that vIRF3 is able to induce apoptosis in TNF-α-treated HEK293T cells via inhibition of NF-κB activity (103). Based on these contradictory find-

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ings, it is not clear whether vIRF3 is pro- or antiapoptotic. The discrepancy may be due to heterologous cell systems studied, overexpression of exogenous vIRF3, and/or the use of different apoptosis-inducible agents. However, recent data from Wies et al. showed that the knockdown of vIRF3 in KSHV-infected PEL (BC-3, JSC-1) cells led to reduced cell proliferation and increased caspase 3/7 activity, demonstrating the antiapoptotic and tumorigenic properties of vIRF3 (89).

**vIRF4 (ORF K10)**

**Transcriptional regulation.** vIRF4 (911 amino acids) is expressed during virus reactivation and serves as a positive coregulator for RTA, the master regulator of the switch from latency to lytic reactivation in KSHV (137) (Fig. 1A and B). Depletion of vIRF4 during TPA-mediated reactivation from latency resulted in a reduced yield of infectious KSHV virions, underlying its role in efficient KSHV reactivation.

**vIRF4 AND CANCER**

**vIRF4 and p53.** Unlike other vIRFs, vIRF4 does not target and antagonize the host IFN-mediated antiviral response. However, it was shown that vIRF-4 interacts with the murine double minute 2 (MDM2) E3 ubiquitin ligase, leading to the reduction of p53 via proteasome-mediated degradation (138) (Fig. 3). The central region of vIRF4 (amino acids 606 to 758) is required for its interaction with MDM2, which leads to the suppression of MDM2 auto-ubiquitination, resulting in a dramatic increase in MDM2 stability. Consequently, vIRF4 expression markedly enhances p53 ubiquitination and degradation, which effectively suppresses p53-mediated apoptosis. Recently, vIRF4 was also shown to specifically inhibit the herpesvirus-associated ubiquitin-specific protease (HAUSP) that regulates the stability of p53 and MDM2. vIRF4 protein binds both the HAUSP TNF receptor-associated factor (TRAF) and the catalytic domains, resulting in the inhibition of substrate binding and HAUSP deubiquitination activity (139). This study showed that two vIRF-4-derived peptides, vif1 and vif2, are able to suppress HAUSP activity and restore p53-dependent apoptosis in PEL cells, as well as suppress tumor growth, in a mouse xenograft model (139). Thus, the virus has developed a unique strategy to target the HAUSP-MDM2-p53 pathway.

**vIRF4 and Notch.** Activated Notch CSL/CFB1 signaling was shown to promote the survival of KSHV-infected cells (140, 141, 142). Notch signaling is an evolutionarily conserved signal transduction pathway which regulates multiple developmental processes. Its deregulation is directly linked to many human disorders, including cancer (143). Notch receptors are transmembrane proteins that upon ligand binding are proteolytically cleaved to generate the intracellular Notch fragment, NICD, which translocates into the cell nucleus and binds to the CSL/CFB1 protein. This DNA-binding factor then recruits corepressor and coactivator complexes to modulate the expression of target genes (143). vIRF4 was shown to act as a potential antagonist of the Notch/CFB1 signal transduction pathway. Interaction of vIRF4 with CBF1 results in inhibition of CBF1-NICD complex formation. These observations were further supported in reporter gene assays, in which vIRF4 interfered with CBF1-dependent Notch transactivation. Thus, it appears that vIRF4 and NICD binding to CBF1 is mutually exclusive and vIRF4 can interfere with NICD-mediated promoter activation via direct competition for CBF1 binding (144).

**CONCLUSION AND PERSPECTIVES**

The critical role of interferon in the innate and adaptive antiviral response has been clearly established. It has also become clear that viruses have evolved mechanisms by which they can attenuate antiviral responses. KSHV has developed multiple redundancies of antiviral proteins to control cellular mechanisms that are involved in immune responses. With the ability to attenuate both arms of the antiviral response, KSHV also hijacked several cellular genes to act as regulators of their cellular homologues. Among these are homologues of the transcription factors of the IRF family, vIRFs (Table 1) (Fig. 1, 2, and 3). The present data indicate that vIRFs function generally as negative regulators of the antiviral response and apoptosis mediated by cellular IRFs.

KSHV and rhesus macaque rhadinovirus (RRV), the two highly related gammaherpesviruses, are the only viruses known to
encode gene products with significant homology to cellular IRFs. Characterization of an RRV recombinant clone lacking all eight vIRFs (vIRF-knockout [vIRF-KO] RRV) demonstrated that RRV-encoded vIRFs inhibit type I and II IFN gene induction (145). Moreover, infection of vIRF-KO RRV resulted in decreased viral loads and diminished B cell hyperplasia, a characteristic pathology of RRV infection (146). Collectively, these findings demonstrate that both KSHV and RRV vIRFs have a broad impact on herpesvirus pathogenesis and host immune responses.

The role of vIRFs in KSHV pathogenesis may extend beyond their immunomodulatory functions. In addition to the previously reported upregulation of the Toll-like receptor 3 (TLR3) pathway in human monocytes during KSHV primary infection, activation of TLR7/8 signaling is important for reactivation of KSHV from latency (147–149). Agonists specific for TLR7/8 reactivated latent KSHV and induced viral lytic gene transcription and replication. Thus, the vIRF-mediated downmodulation of TLR signaling and its effector molecules, IFNs and NF-κB, may also prevent KSHV reactivation and serve as an important control mechanism for the latent-to-lytic switch.

In addition to their immunomodulating effects, KSHV-encoded viral IFNs were also shown to modulate cell growth by targeting the function of the tumor suppressor p53 and enhancing the activity of the c-Myc proto-oncogene. Since p53 is rarely mutated in KSHV-associated tumors (150), the inhibition of p53-mediated signaling appears to be a key regulatory pathway through which KSHV is able to establish malignancy. Supporting this finding, chemical activation of p53 by an MDM2 antagonist, Nutlin-3, in PEL cells led to unimpaired induction of p53 target genes as well as growth inhibition and apoptosis (151).

Although different vIRFs often target the same cellular pathways, their specificity and redundancy is yet to be determined. The recent work of Jacobs and colleagues attempted to shed light on the differences in the mechanisms through which vIRF1, -2, and -3 could inhibit TLR3-mediated activation of IFN-β. Although all three vIRFs were able to inhibit TLR3-mediated activation of IFN transcription reporters, only vIRF1 and -2 inhibited IFN-β message and protein levels (152). Furthermore, the expression of vIRF1 seemed to reduce IRF-3 phosphorylation and nuclear localization compared to results for vIRF2, suggesting that while both vIRF1 and vIRF2 inhibit TLR3-mediated induction of IFN-β, they may accomplish this via distinct mechanisms. The advantage of the redundancy of the vIRFs in the antiviral response is easy to understand. However, it is not clear why KSHV evolved these vIRFs to also control so many growth regulatory and antiapoptotic functions. One has to wonder if the multiple roles of vIRFs in the modulation of cellular growth and apoptosis mirror the multiple ways in which KSHV uses cellular machinery for its own replication and the establishment and maintenance of latency. Future studies will undoubtedly answer some of these questions.

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