Regulation of growth signalling and cell cycle by Kaposi’s sarcoma–associated herpesvirus genes

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Summary
Kaposi’s sarcoma-associated herpesvirus (KSHV) is the primary aetiological agent of at least three malignancies associated with HIV infection and immunosuppression: Kaposi’s sarcoma, primary effusion lymphoma and multicentric Castleman’s disease. KSHV encodes proteins that deregulate key checkpoints in the signalling pathways governing cell proliferation, which may ultimately contribute to the virus’ oncogenic potential. To alter cellular signalling associated with proliferation, these viral proteins function like growth factor ligands/receptors, signal transduction proteins, transcription factors and cell cycle regulators. This review focuses on the mechanisms by which some KSHV-encoded proteins activate signalling pathways and cell proliferation and their role in the pathogenesis of KSHV-driven mechanisms.

Keywords
cell cycle, chemokines, Kaposi’s sarcoma, oncogenesis, signal transduction, transcription factors

Kaposi’s sarcoma–associated herpesvirus (KSHV)
Kaposi’s sarcoma (KS) was originally described as an indolent disease mainly in elderly Mediterranean and African populations, as well as in transplant recipients. Interest in KS soared with its increased prevalence as an aggressive cancer associated with HIV and AIDS. Supported by compelling epidemiological studies, an infectious agent was sought and subsequently discovered in AIDS-KS lesions by representational difference analysis (Chang et al. 1994). Kaposi’s sarcoma-associated herpesvirus, KSHV, the eighth human herpesvirus (HHV8), is a member of the γ2-herpesviridae subfamily and is most closely related to the oncogenic simian virus, Herpesvirus saimiri (HVS). The closest related HHV is the oncogenic herpesvirus, Epstein–Barr Virus (EBV or HHV-4), a γ1-herpesvirus (Neipel et al. 1998). KSHV infection is also associated with two rare lymphoproliferative disorders, primary effusion lymphoma (PEL) (Cesarman et al. 1995) and multicentric
Castleman’s disease (MCD) (Soulier et al. 1995), both of which are tumours of the B-cell lineage and are also seen more frequently in HIV-infected individuals.

The multiple pigmented vascular lesions of KS are characterized by spindle-shaped cells, which are derived from lymphatic endothelial cells, and are infiltrated by inflammatory cells (Boshoff & Weiss 2002; Wang et al. 2004a). The vast majority of spindle cells, found in the later nodular stage of KS, express a latent profile of KSHV gene expression, where only a fraction of the total KSHV genes is expressed. Many more KSHV genes are expressed in lytically infected cells, which constitute 1–5% of the total infected cells in KS lesions. The KSHV genome encodes a number of genes with cellular homologues, suggesting a high degree of capture of host genes, not seen with any other herpesvirus. These viral genes have homology to cellular growth factors, angiogenic factors, anti-apoptotic proteins, transcription factors and cell cycle regulators, and are capable of disrupting normal growth signalling and regulatory checkpoints in cell proliferation as observed in cancer in general. This review focuses on both latently and lytically expressed genes of KSHV which are suspected to contribute to its pathogenicity due to their ability to stimulate cellular proliferation by affecting known signalling pathways. Both lytic and latent KSHV-encoded genes demonstrate the ability to activate the growth signalling by functioning as growth factors, growth factor receptors, signal transduction molecules, transcription factors and critically, cell cycle regulators. Often these viral proteins are homologous to cellular proteins, but unlike their cellular counterparts, they cannot be fully regulated, and thus, constitutively activate growth signalling and allow unchecked cell proliferation.

For the purposes of this review, KSHV genes known to affect growth signalling have been divided into: growth-factor receptor ligands and growth-signalling membrane proteins/ signal-transduction proteins. The review will also cover known angiogenic factors encoded by KSHV, as activation of angiogenesis can also be considered as promoting tumour growth. Finally, KSHV-encoded transcription and cell cycle regulators that directly impinge on cell proliferation will be discussed.

KSHV-encoded growth-factor receptor ligands and signalling proteins

The initiation of cell proliferation is determined by the extracellular environment, where the presence of growth factors is one determinant. The synthesis and binding of growth factors to cognate receptors initiates signalling cascades which culminate in the expression of genes capable of promoting cell proliferation. However, tumour cells demonstrate the ability to initiate cell proliferation independently of ‘normal’ stimuli through constitutive activation of growth-promoting signalling pathways. Such sustained signalling may result from tumour cells overexpressing growth receptors and/or their ligands to enhance tumour-cell sensitivity to proliferative signals or the expression of constitutively-activated receptors or signal-transduction proteins, whose activity is independent of ligand binding.

Similar mechanisms are thought to be at work in KSHV-infected tumour cells that express viral proteins with homology to growth-factor receptors/ligands as well as signal transduction proteins. In addition, the virus encodes ‘unique’ membrane-bound proteins, which function as constitutive signalling receptors. This section of the review discusses these KSHV-encoded proteins and the signalling pathways regulated by them.

KSHV-encoded growth-factor receptor ligands: viral interleukin-6 (vIL-6)

One characteristic of patients with KSHV-driven B-cell lymphomas, PEL and MCD, is high serum levels of human IL-6 (hIL-6). KSHV encodes an open reading frame (ORF) K2 or vIL-6, a homologue of hIL-6 which is expressed in both lytic and latently infected cells (Moore et al. 1996; Neipel et al. 1997). Signalling through the hIL-6 receptor requires the ligand-dependent assembly of a complex consisting of the IL-6 receptor (α-subunit) and an additional membrane protein, gp130 (the β-subunit), which homodimerizes to permit signalling (Figure 1). The specificity of hIL-6 signalling is regulated by the limited availability of the α-subunit, which binds the hIL-6 ligand, while the β-subunit is more ubiquitously expressed. The downstream transducers of IL-6 receptor signalling are the Ras/MAPK and Jak/STAT pathways, which mediate transcriptional activation through IL-6-response elements in the promoters of genes like c-fos and c-jun. Many B-cell tumour lines are dependent on IL-6 signalling for growth. In such cells, v-IL6 can replace hIL-6, demonstrating functional homology between the viral and cellular IL-6 (Moore et al. 1996; Burger et al. 1998). In contrast to hIL-6, vIL-6 can signal through gp130 in the absence of the α-subunit (Wan et al. 1999; Aoki et al. 2001a; Li et al. 2001). As a result, v-IL6 can be a ubiquitous signal inducer, stimulating signalling in cells expressing only gp130 (Hoischen et al. 2000; Mullberg et al. 2000). Like the hIL-6, vIL-6 utilizes Ras/MAPK and JAK/STAT pathways, constitutively activating them (Osborne et al. 1999; Hideshima et al. 2000). In KSHV biology, vIL-6 has a multifunctional role in cell proliferation, oncogenic transformation and host immune responses. The importance of IL-6 in KSHV-associated B-cell-lymphomas proliferation has been demonstrated.
PEL-derived cell lines show growth dependency on vIL-6 and IL-10 and induce the expression of hIL-6 (Jones et al. 1999). Moreover, the capacity of vIL-6 to directly transform cells and potentially contribute to KSHV-mediated oncogenesis has been demonstrated in tumour formation assays in mice (Aoki et al. 1999). vIL-6 also overcomes interferon-α (IFN-α)-mediated anti-proliferative cellular responses induced by viral infection. The host antiviral IFN-α response induces the transcription of cell cycle inhibitor p21 and downregulates the expression of the IL-6 receptor α-subunit, both contributing to inhibition of cell proliferation. vIL-6 overcomes this anti-proliferative response by blocking p21 transcription and bypassing downregulation of the α-subunit by signalling solely through the β-subunit. Remarkably, the KSHV genome also ensures increased expression of vIL-6 in the context of an antiviral response with IFN-α-induced transcription of vIL-6 via two IFN-stimulated response elements within the vIL-6 promoter (Chatterjee et al. 2002). The increased expression of vIL-6 in response to IFN-α is also seen when lytic replication is induced in PEL cell lines, hinting at a close interplay between host immune responses and viral replication (Chatterjee et al. 2002). The fact that PEL and MCD patients frequently demonstrate high serum levels of hIL-6, coupled with findings that vIL-6 is transforming and is required for PEL cell growth suggests that IL-6 signalling is critical for development of these malignancies (Aoki et al. 2001b; Aoki et al. 2001c). Mouse models also support a role for IL-6 in oncogenesis where overexpression of IL-6 from bone marrow cells induces a pathology strikingly similar to MCD (Brandt et al. 1990). Moreover, several other KSHV genes contribute to increased expression of IL-6, such as latency associated nuclear antigen-1 (LANA-1) (An et al. 2002), vFLIP (An et al. 2003b) and viral G-protein-coupled receptor (vGPCR) (Polson et al. 2002; Montaner et al. 2004), inducing IL-6 expression via activation of the jun and nuclear factor-kappa B (NF-κB) signalling pathways (Figure 1). The use of IFN-based therapies to limit IL-6 signalling as well as anti-IL6 monoclonal antibodies are proposed therapies for PEL and MCD (Asou et al. 1998; Drexler et al. 1999).

KSHV-encoded growth-factor receptor ligands: viral macrophage-inhibitory proteins (vCCL1, vCCL2 and vCCL3)

The deregulated expression of chemokines is associated with tumour growth, angiogenesis and metastasis, which are partly due to constitutive activation of the NF-κB pathway, which potentiates chemokine transcription in a positive feedback loop (Richmond 2002). Chemokine signalling is mediated through seven transmembrane domain GPCRs that activate a multitude of growth signalling pathways including phospholipase-Cβ (PLC-β), phosphatidylinositol 3-kinase (PI-3K), mitogen-activated protein kinase (MAPK)/extracellular response kinase (ERK), Rho, p21-activated kinase, p38 MAPK and NF-κB. The α and β herpesviruses including Marek’s disease virus, cytomegalovirus, HHV6 and HHV7 deregulate the chemokine system through mimicry, encoding chemokine homologues and/or putative chemokine receptors (Murphy 2001).

KSHV encodes chemokine homologues vCCL1, vCCL2 and vCCL3 encoded by ORF K6, ORF K4 and ORF K4.1, respectively, as well as a putative chemokine receptor, vGPCR. The cellular counterparts of the KSHV chemokines, the human macrophage-inhibitory proteins (MIPs) are members of the CCL family of chemokines of which vCCL1 and vCCL2 most closely resemble MIP-1α. In fact, vCCL1 and vCCL2 are more homologous to one another than with any cellular chemokine, suggesting that they are derived from a gene duplication event during the evolution of the virus (Moore et al. 1996).

Owing to the vascular nature of KS, the vCCLs were hypothesized as possible angiogenic factors for these tumours. Mechanistically, angiogenesis resulting from viral chemokines can either be induced through direct signalling or possibly by chemotactic properties of vCCL2, recruiting of leucocytes, monocytes and macrophages to sites of inflammation where they produce pro-angiogenic factors. This indirect model is supported by the finding that KS lesions are infiltrated by leucocytes. Although vCCL1, vCCL2 and vCCL3 possess no sequence homology to known angiogenic chemokines, CXCL8 (IL-8) and CXCL1 [growth related oncogene-α (GRO-α)], they demonstrate angiogenic properties in chick chorioallantoic membrane assays. This contrasts their cellular homologues, MIP-1α and RANTES, which failed to induce angiogenesis in the same assays (Boshoff et al. 1997; Stine et al. 2000). One additional mechanism for vCCL1-mediated angiogenesis involves induction of a potent angiogenic factor, vascular endothelial growth factor-A (VEGF-A) and its receptor VEGFR1 (Flt-1), which was observed in vCCL1-treated PEL cells (Liu et al. 2001). This allows for autocrine stimulation by vCCL1, inducing angiogenesis; the viral chemokine triggers the expression of cellular angiogenic factors by KSHV-infected tumour cells, while simultaneously enhancing the host cell’s responsiveness by increasing the expression of the cognate receptor, FH-1. Within the microenvironment of an angiogenic KS lesion, upregulation of VEGF signalling and responsiveness may enhance proliferation of the tumour cells. Chemotactic properties of the vCCLs may further enhance angiogenesis and tumour growth through recruitment of monocytes/macrophages to KS tumours to produce angiogenic factors. This may be an additional indirect mechanism of angiogenesis, although whether this can be attributed to...
the chemotactic properties of the vCCLs or other KSHV genes has yet to be determined.


In addition to the viral chemokines, KSHV-mediated subversion within the chemokine system also extends to a viral chemokine receptor. The vGPCR is a lytic gene of KSHV encoded by ORF74. It is homologous to the human chemokine receptors CXCR1 and CXCR2, which bind angiogenic chemokines CXCL1 and CXCL8 (Cesarman et al. 1996; Kirshner et al. 1999). vGPCR was found to be a transforming viral protein in tumour formation assays in mice (Arvanitakis et al. 1997; Bais et al. 1998). vGPCR as a potential viral oncogene in KS is also supported by mouse models, where mice develop multifocal KS-like lesions when vGPCR expression is restricted to haematopoietic or endothelial cells (Yang et al. 2000; Montaner et al. 2003). Unlike its human homologue, vGPCR signals constitutively; requiring no ligand binding (Arvanitakis et al. 1997; Bais et al. 1998). Interestingly, mutation of a conserved DRY amino acid sequence to VRY, as found in vGPCR, was sufficient to induce constitutive signalling of CXCR2 and induce oncogenic transformation (Burger et al. 1999). This supports the idea that signalling through vGPCR is a transforming activity of KSHV.

Like the cellular chemokine receptors, vGPCR is predicted to have seven transmembrane domains, with conserved glycosylation sites in its N-terminus and cysteine residues in the

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**Figure 1** Enhanced interleukin-6 (IL-6) signalling by Kaposi’s sarcoma-associated herpesvirus (KSHV) proteins. The presence of high serum levels of IL-6 is a characteristic of the KSHV-driven lymphomas and suggests that tumour cell proliferation is dependent on IL-6 signalling. KSHV proteins enhance IL-6 signalling, by direct and indirect mechanisms through their effects on the IL-6 receptor. Unlike its cellular counterpart, viral interleukin-6 (vIL-6) can directly initiate IL-6 signalling, independently of IL-6 receptor expression. Other KSHV proteins, viral G-protein-coupled receptor (vGPCR), vFLIP-1 and latency associated nuclear antigen-1 (LANA-1) induce transcriptional activation of the hIL-6 gene via JNK/SAPK and NF-κB pathways. In co-operation with vIL-6 expression from KSHV-infected cells, the high serum IL-6 contributes to enhanced signalling.

extracellular loops. Although constitutively active, vGPCR signalling can be modulated by both the human and viral chemokine agonists and inverse agonists which include, CXCL1, CXCL8 and vCCL2 (Geras-Raaka et al. 1998; Gershengorn et al. 1998). The ability of the vGPCR to signal in a ligand-dependent and -independent fashion may suggest modulation of the signalling profile of vGPCR in the microenvironment of KSHV tumours, which may contribute to tumourigenesis and/or viral replication.

Heterotrimeric G-proteins are comprised of an α-subunit and a βγ-heterodimer that bind to GPCRs. Activation of the GPCR induces an exchange of guanosine diphosphate for guanosine triphosphate and results in a conformational change freeing the α-subunit and the βγ-heterodimer to activate downstream-signalling effector proteins. The broad signalling capability of vGPCR can be attributed to its use of several G-protein α-subunit families Gz₁, Gz₂ and Gz₁₃ to signal to distinct cellular pathways (Shepard et al. 2001; Smit et al. 2002). In addition, the Rho-family of monomeric G-proteins, specifically Rac-1, has also been shown to mediate the signalling of vGPCR (Montaner et al. 2004). The genes activated by vGPCR in experimental models, are those under the regulation of NF-κB, activator protein-1 (AP-1), cAMP response element binding protein (CREB) and nuclear factor of activated T cells (NFAT). The signalling effectors activated are similar to those induced by pro-inflammatory chemokines and include PLC, PI-3K as well as the SAPK and MAPK cascades (Montaner et al. 2001; Shepard et al. 2001; Smit et al. 2002; Cannon et al. 2003; Pati et al. 2003).

The signalling pathways induced by vGPCR are similar to those of its human homologue CXCR2, a chemokine receptor associated with angiogenesis in a number of tumours. vGPCR, in addition to K1 and v-IL6 have also been shown to increase the expression of angiogenic VEGFs levels of which are also significantly elevated in HIV-positive individuals, further implicating a role for VEGF in the development of KSHV-driven tumours. vGPCR-induced angiogenesis through enhanced VEGF expression, appears to be mediated by activation of a MAPK/hypoxia-induced factor-1α pathway (Sodhi et al. 2000). Additional mechanisms of angiogenesis may also include vGPCR upregulation of angiogenic chemokines, IL-8 and GRO-α. Although vGPCR is an early lytic gene, a 'hit and run' mechanism has been proposed for its function as a viral oncogene (Bais et al. 2003). vGPCR oncogenesis may be mediated by enhanced expression of VEGF and VEGFR2 to drive cell proliferation in a paracrine manner. In human umbilical vascular endothelial cells, one experimental model for the KSHV-infected cells in KS, expression of vGPCR promotes cell survival and induces immortalization (Montaner et al. 2001; Bais et al. 2003). Further characterization of vGPCR-induced sarcomas in mice correlates with high activity of Akt/protein kinase B, a downstream kinase of vGPCR signalling. Pharmacological inhibition of Akt in these mice reduced the tumourigenic potential of vGPCR, suggesting that activation of this pathway is a significant factor in the development of vGPCR-induced sarcomas and perhaps KS (Sodhi et al. 2004).

Although transiently expressed in lyrically infected cells, which make up a small number of the infected cells of the total KSHV-infected tumour, a role of vGPCR in the pathogenesis of KS maybe to induce expression of angiogenic factors, such as VEGF, IL-8 and GRO-α, which stimulate cell proliferation and provide an environment more conducive to oncogenic transformation of latently infected cells.

**KSHV-encoded growth-signalling membrane proteins: K1**

Over 80 ORFs have been characterized for KSHV and of these, 15 ORFs are unique to the virus with no identified cellular homologues and have been given the prefix K. ORF K1 is one such unique gene identified through the sequencing of the terminal repeat region of the KSHV episome (Lagunoff & Ganem 1997). The K1 gene shares the same genomic locus to the simian-transforming protein of related virus, HVS and latent membrane protein 1 (LMP1) of EBV, but shares no homology with any known viral or human protein. Initial characterization of the K1 transcript showed an early lytic gene expression pattern, and it was predicted to encode a membrane-bound protein with a hydrophobic N- and C-terminus.

Further analysis of the K1 amino acid sequence identified a number of immunotyrosine-activation motifs (ITAMs) (Lee et al. 1998a). ITAMs are conserved tyrosine residues located on the cytoplasmic tail of activating receptors that modulate interaction with other adaptor proteins involved in signal transduction. These tyrosine motifs are targeted for phosphorylation by protein tyrosine kinases such as the Src family of kinases that are recruited to the stimulated B-cell receptors (BCR). These phosphorylated tyrosine residues can serve specifically as sites of interaction for signal transduction proteins carrying SH2 domains. ITAMs are essential in the activation of receptor-mediated cell signalling on stimulation by a ligand and are associated with the signalling capabilities of BCR as well as T-cell receptors.

The phosphorylation of tyrosine residues in the ITAM of K1 has been shown to be important in the subsequent phosphorylation and activation of signal transduction proteins syk, cbl and vav, as well as activating NFAT by a phospholipase Cγ₂ (PLCγ₂) pathway and the p85 subunit of PI-3K (Lagunoff et al. 1999). Further characterization of PI-3K activation by K1 has revealed that Akt is concomitantly activated to inhibit apoptosis (Tomlinson & Damania 2004). The activation of these multiple signalling pathways may contribute to the
oncogenic potential of K1 as shown by tumour formation in mice by K1-transformed rat embryonic fibroblasts and K1-transgenic mice (Lee et al. 1998b; Prakash et al. 2002). Although expression of K1 was not restricted to a particular cell lineage in the transgenic mice, they developed malignant plasmablastic lymphomas, as seen in PEL, suggesting that the oncogenic effects of K1 are evident only in KSHV-driven lymphomas. More recently, the effects of K1 in endothelial cells have been tested, where K1 expression induced VEGF and invasion factor, matrix metalloprotease-9 expression (Wang et al. 2004b). Although K1 activates a number of signalling pathways able to induce transformation, similar to vGPCR, its role in KSHV oncogenesis may involve enhancing the tumorigenicity of latently infected cells in a paracrine fashion, by K1-induced expression of growth and invasive factors.

KSHV-encoded growth-signalling membrane proteins: Kaposin

The KSHV-encoded protein, Kaposin, was characterized as a transforming gene encoded by ORF K12, expressed in latently infected PEL cells (Muralidhar et al. 1998). Initial characterization revealed the protein localized to the Golgi apparatus and had the ability to activate kinases involved in cell signalling (Muralidhar et al. 2000). However, further analysis of Kaposin transcription and translation revealed three isoforms of the protein: Kaposin A, which is translated from ORF K12, as initially characterized, and Kaposin B and Kaposin C, which are translated from a larger transcript-utilizing sequences upstream of ORF K12. All three forms are detected in KS and PEL cells (Sturzl et al. 1997; Sadler et al. 1999; Li et al. 2002).

The role of Kaposin A as a membrane-bound regulator of cell-signalling pathways is controversial due to conflicting data on the localization of the protein isoforms. Kaposin A has most recently been described as having a perinuclear distribution distinct from that of the Golgi apparatus (Tomkowicz et al. 2002). An earlier study shows the presence of Kaposin A in membrane fractions. Kaposin A interacts with and activates cytohesin-1, a guanine nucleotide exchange factor involved in integrin signalling (Kliche et al. 2001). A dominant-negative cytohesin-1 blocked activation of an ERK2/AP-1 pathway and reduced Kaposin A-induced focus formation.

Although Kaposin A represents a potential viral oncogene, little is known about its role in deregulating cell signalling. The interaction of Kaposin A with cytohesin-1 suggests that a novel mechanism involving the deregulation of integrin signalling contributes to Kaposin-mediated transformation, however further study of Kaposin and its isoforms may reveal other mechanisms.

KSHV-encoded growth-signalling membrane proteins: K15

In addition to ORF K1, KSHV encodes another unique receptor-like protein at the extreme right end of the KSHV genome named K15. ORF K15 encodes a protein of predicted mass of about 50 kDa, although 35 kDa and 23 kDa species are the predominant forms of the protein expressed in PEL cells. Two divergent alleles known as the predominant and minor forms encode the K15 proteins, which share a common C-terminal cytoplasmic domain (Poole et al. 1999). Further analysis of the K15 transcript also reveals expression of several splice variants that are most highly expressed during the induction of lytic replication (Choi et al. 2000). Further modification of K15 protein at the translational level is also predicted by identification of putative protein cleavage sites within the K15 amino acid sequence. K15 expression has been reported in latently infected PEL and MCD cells (Sharp et al. 2002).

Although K15 has no human homologue, it most closely resembles the LMP2A of EBV in its location in the viral episome, its protein motifs and function, which may indicate co-evolution of the two viral genes. Like K15, LMP2A is located at the far right end of the viral episome and encodes two putative SH2-interacting domains that resemble those found on K15. The putative TNF-α receptor-associated factor (TRAF)-binding domain of K15 is similar to the oncogenic protein LMP1 of EBV, which functions like human CD40 in the activation of NF-κB signalling by BCR. The similarity in protein-binding motifs suggests that some of the signalling pathways used by LMP2A and LMP1 could also be activated by K15. This potential membrane cell signalling function for K15 is further suggested by its predicted structure, consisting of a N-terminus extracellular domain linked to 12 transmembrane domains and a C-terminus cytoplasmic tail containing putative ITAMs (YEEVL and YASIL) and an SH3 protein-interaction motif. Also located on the C-terminus is a TRAF domain, an interaction domain for the TRAF signal transduction proteins and activators of AP-1 and NF-κB pathways. The signalling potential of K15 has been revealed with many of the signal-activating functions being attributed to the YEEVL ITAM. This motif is phosphorylated by the B-cell-signalling tyrosine kinases Src, Lck and Fyn to activate the MAPK cascade and consequently AP-1 by the ERK2 and JNK-1 pathways. The YEEVL region interacts with TRAF2. It has been shown that the downstream activation of AP-1 can be inhibited by a TRAF2-dominant negative, suggesting that K15 acts through a TRAF2-dependent signalling pathway. The YEEVL motif of K15 is also required for the activation of NF-κB as demonstrated in gene reporter assays. The activation of AP-1 and NF-κB pathways and localization of K15 to lipid

rafts involved in signal transduction together indicate that K15 is a membrane-bound signalling molecule with functional homology to LMP1 and LMP2A of EBV (Brinkmann et al. 2003). Functional homology between the LMPs of EBV and K15 also extends to deregulation of other B-cell-signalling pathways. Like LMP2A, K15 can mimic BCR signalling by providing survival signals and has been shown to enhance the survival of BCR-deficient B-cell lineages. Although K15 has been shown to activate pathways inducing cell proliferation, there is currently no data addressing its transforming ability. The EBV homologue LMP2A is not essential for EBV-induced cellular transformation, but it has a role in epoxide maintenance, protecting latently infected cells from lytic activation induced by host immune responses. The latter function may be fulfilled by K15 in KSHV latency.

**KSHV-encoded signal transduction protein: viral FLICE inhibitory protein (vFLIP)**

The viral FLICE [Fas-associated death domain (FADD)-like IL-1β-converting enzyme] inhibitory protein (vFLIP) is a homologue of the cellular FLIP (cFLIP) protein, and it is encoded by ORF71 of the viral episome. This ORF is co-ordinately transcribed with two other latently expressed genes, cyclin and LANA-1. These three genes are transcribed from a single promoter producing a tricistronic mRNA from which the LANA-1 transcript is spliced (Talbot et al. 1999). An internal ribosome entry site links the translation of both cyclin and vFLIP.

cFLIP is an inhibitor of death receptor signalling from receptors like tumour necrosis factor receptor 1 (TNFR1) and Fas ligand receptor. cFLIP functions by blocking recruitment of caspase 8 to the death-induced signalling complex (DISC) which assembles at the death receptor with receptor-associated adaptor proteins upon ligand binding. Procaspase 8 is normally recruited to DISC via death effector domains (DED), which are also found in cFLIP. vFLIP also contains DEDs which allow it to function like its cellular homologue, preventing cleavage of procaspase 8 to its active form, thus inhibiting Fas-mediated cell death (Djerbi et al. 1999; Belanger et al. 2001).

cFLIP has an anti-apoptotic function via activation of the NF-κB-signalling cascade by the death receptors such as TNFR1. TNFR1 is able to activate NF-κB through interaction with NIK and RIP proteins of the NF-κB-activation cascade and their association with the receptor adaptor proteins TRAF2 and FADD. The activation of NF-κB by TNFR1 is through a kinase cascade activated by ligand binding to the receptor and culminates in the assembly of the NF-κB signalosome, which comprises inhibitory kappa kinases (IKKa, IKKβ and IKKy). This active kinase complex in turn phosphorylates and targets for proteasomal degradation of the IκB protein, which frees the NF-κB-transcription factors to translocate to the nucleus and activate transcription of NF-κB-responsive genes. Also activated in parallel with NF-κB is the JNK/SAPK pathway via a TRAF2-dependent mechanism. It was postulated that vFLIP would share functional homology to cFLIP and activate the same pathways, and subsequently vFLIP was shown to activate transcription from NF-κB-response elements through persistent activation of the NF-κB signalosome, with which vFLIP is in complex (Chaudhary et al. 1999; Liu et al. 2002; Field et al. 2003). vFLIP can also upregulate expression of NF-κB precursor protein p100 and enhance its processing to the transcriptionally active p52 form, an alternative pathway of NF-κB activation (Matta & Chaudhary 2004) as well as activating the parallel JNK/SAPK pathway to induce the expression of hIL-6, which can also be modulated by vFLIP/NF-κB (An et al. 2003b).

The presence of high NF-κB activity is associated with many lymphoproliferative disorders and seems to be essential for their continued growth and survival. Cell lines derived from PEL also demonstrate dependency on high constitutive activity of NF-κB as suggested by the presence of high levels of phosphorylated IκB and decreased survival of PEL cells when NF-κB is pharmacologically inhibited (Keller et al. 2000; Liu et al. 2002 Field et al. 2003). The presence of high NF-κB activity may be attributed in part to the action of vFLIP. A recent study using targeted knock down of vFLIP by RNA interference (RNAi) in PEL cells has demonstrated that the expression of vFLIP is essential to the survival of PEL cells, suggesting vFLIP activation of NF-κB is critical for PEL-cell survival (Gusparri et al. 2004). This coupled with studies of the oncogenic potential of vFLIP in transformation and tumour-formation assays (Matta et al. 2003) have proposed targeting of vFLIP and the NF-κB pathway in PEL by RNAi and/or pharmacological inhibitors of NF-κB activation as a therapeutic approach to the treatment of KSHV lymphomas.

**KSHV-encoded transcription and cell cycle regulators**

A majority of somatic cells are in a noncycling state termed G0, or quiescence, but can re-enter the cell cycle in response to external signals to proliferate. Crucial regulators of re-entry into the cell cycle are the cyclin/cyclin-dependent kinase (cdk) complexes. Many of the signalling pathways that convey the external signals to proliferate converge on the transcription of D-type cyclins and their assembly with catalytic subunits, cdk4 and cdk6. One substrate for cyclin D/cdk complexes is the retinoblastoma protein (pRb), which functions to relieve pRb interaction with the E2F-transcription factor, whose transcriptional targets enable entry to S-phase of the cell cycle. Cyclin/
cdk complexes interact with two families of cyclin-dependent kinase inhibitors (CDKIs): the inhibitor of cdk4 (INK4) family (p15, p16, p18 and p19) which prevent complex assembly and the Cip/Kip/Waf family (p21, p27 and p57) which aid assembly and nuclear import of cyclin D/cdk complexes but conversely inhibit other cyclin/cdk complexes to arrest the cell cycle (Planas-Silva & Weinberg 1997; Sherr 2000).

Frequently genes in the cyclin D/pRb/cdk/INK4a pathway are mutated in cancer, resulting in aberrant cell proliferation, aiding tumourigenesis (Hall & Peters 1996). Other proteins that arrest cell cycle or cause apoptosis are also frequently mutated in tumours, especially pRb and the p53 tumour suppressor gene, an activator of cell cycle arrest (via p21 Cip) and apoptosis. KSHV proteins that promote cell cycle progression and that inactivate tumour suppressor genes are hypothesized to be contributory factors in viral deregulation of cell cycle and promoting oncogenesis (Figure 2).

This section of the review focuses on KSHV mechanisms of cell cycle deregulation through transcriptional regulation and viral protein interactions with cell cycle regulators.

**Figure 2** Regulation of the G1 cell cycle restriction point by Kaposi’s sarcoma-associated herpesvirus (KSHV) proteins: Re-entry into cell cycle is regulated by the activity of the D-type cyclin/cdk complexes during the G1 phase of the cell cycle. Signalling from KSHV-encoded growth factor receptor ligands and signalling proteins culminate in the synthesis of D-type cyclins, which assemble with cdk4 or -6. Further, the viral interferon regulatory factor-1 (vIRF-1), viral interleukin-6 (vIL-6), latency associated nuclear antigen-1 (LANA-1) and vcyclin bypass or inhibit the cyclin-dependent kinase inhibitors (CDKIs) to enhance activity of the cellular cyclin/cdk complexes. CDKI-resistant vcyclin/cdk6 complexes phosphorylate and inactivate the retinoblastoma protein (pRb) to permit transcription of E2F-regulated genes and allow entry into S-phase. Rb inactivation is further augmented by LANA-1 interaction with Rb.

KSHV-encoded transcription and cell cycle regulators: viral interferon regulatory factors (vIRFs)

Viral infection results in the activation of the host immune system and evokes cell responses to prevent viral replication and to remove the virus by inhibiting cell proliferation and/or killing infected cells. KSHV encodes genes that interfere with many of the host antiviral responses, which sustain viral life cycle and perhaps inadvertently, promote oncogenesis.

One of the earliest responses to viral infection by cells is the expression of the type I IFNs (IFN-α and IFN-β) that result in the expression of genes that cause cell cycle arrest and apoptosis. These genes are upregulated by IFN-regulatory factors (IRF), a family of transcription factors, which are activated by IFN signalling through their cognate receptors (Taniguchi et al. 2001).

KSHV encodes three homologues of the IRFs, vIRF-1, vIRF-2 and v-IRF-3, which regulate the transcriptional activity of the cellular IRFs. The best characterized of these is vIRF-1, which is encoded by ORF K9 and has homology to cellular IRF-8 and IRF-9 (Moore et al. 1996). vIRF-1 has no functional homology to its cellular counterparts, rather, it blocks the
transcription of IRF-1-regulated genes (Zimring et al. 1998) and inhibits the anti-proliferative responses of IFN-γ (Flowers et al. 1998). Furthermore, it has been shown that vIRF-1 is oncogenic, as fibroblasts stably expressing cells vIRF-1 exhibited a transformed phenotype and established tumours in mice (Gao et al. 1997; Li et al. 1998). The oncogenic potential of vIRF-1 may result from its role as an immune regulatory protein, suppressing IFN responses during viral infection to subvert cell cycle control and apoptosis. vIRF-1 also has transcriptional activator properties (Roan et al. 1999), which may also contribute to transformation by regulating the transcription of viral genes (Li et al. 1998).

Though vIRF-1 has similar DNA-binding domains to IRF-1, vIRF-1 has not been shown to directly interact with IRF-DNA-binding elements. A mechanism of repression of IRF-1 may be through interaction with the p300/CREB-binding protein (CBP) transcription complex. vIRF-1 has been found to interact with p300 CBP which inhibits its transactivating potential in gene-reporter assays (Li et al. 2000; Seo et al. 2000). This interaction has been shown to block the recruitment of p300/CBP to cellular IRF-3 and hence, inhibit transcription mediated by IRF-3 (Lin et al. 2001).

An additional mechanism of vIRF-mediated subversion of cell cycle arrest is the inactivation of the p53 tumour suppressor. Both vIRF-1 and vIRF-3 (also known as LANA-2) bind to p53 to inhibit apoptosis (Nakamura et al. 2001; Seo et al. 2001; Rivas et al. 2001), a function that could potentiate vIRF-mediated oncogenesis.

**KSHV-encoded transcription and cell cycle regulators:**

**LANA-1**

LANA-1 is a KSHV gene with no cellular homologue, however, LANA-1 has a viral homologue at the same gene locus, ORF73, in HVS and at the equivalent position to the EBNA-1 gene in the EBV genome. In the viral life cycle, many functions have been attributed to LANA-1 such as KSHV episome maintenance through its interaction with histone H1 (Ballestas et al. 1999), episome replication in dividing cells (Hu et al. 2002; Lim et al. 2002) and more recently described, LANA-1 inhibits the activation of the lytic gene programme by repressing the transcriptional activity of Rta, a KSHV gene which activates lytic replication (Lan et al. 2004). Many of the functions of LANA-1 have been ascribed to its regulation of gene transcription. LANA-1 interacts with a number of transcription-regulatory factors involved in both transcriptional repression and activation. Transcriptional repression by LANA-1 is mediated by its interaction with mSin3A and ATF4/CREB2 (Krithivas et al. 2000; Lim et al. 2001), which modulates both cellular and viral gene transcription. The transcriptional activation properties of LANA-1 are mediated through the C-terminus of the protein, a region responsible for the binding of several transcription regulatory proteins including pRB, really interesting new gene 3 (RING3) (Platt et al. 1999) and the CBP (Lim et al. 2001).

LANA-1 expression affects cell cycle regulation through interaction with the tumour suppressor genes p53 and pRB, repressing p53 to inhibit apoptosis (Friborg et al. 1999) and inactivating Rb to enhance E2F activity (Radkov et al. 2000). These functions of LANA-1 may contribute to oncogenic transformation as demonstrated by LANA-1 co-operation with oncogenic H-Ras in transformation of REF cells (Radkov et al. 2000). E2F activation may be further enhanced by LANA-1 interaction with RING3, a protein also implicated in E2F activation. RING3 also phosphorylates LANA-1, and although the functional significance is not yet known, it is possible that they co-operate in the activation of E2F.

Recently, a novel mechanism for LANA-1 deregulation of the cell cycle was described involving interaction of LANA with glycogen synthase kinase-3β (GSK-3β). GSK-3β is a regulator of β-catenin, a protein involved in the transcription of proto-oncogenes CCND1 (cyclin D1), myc and jun, genes potentiating growth signalling and cell cycle. GSK-3β phosphorylates β-catenin and targets it for degradation via the proteasome. LANA-1 interacts with GSK-3β through its C-terminus, relocating it to the nucleus and preventing its phosphorylation of β-catenin (Fujimuro et al. 2003). In KS samples and PEL-cell lines, β-catenin is stably expressed in the nucleus, suggesting this is a significant mechanism for transformation in KSHV-driven neoplasias (Fujimuro & Hayward 2003). LANA-1 is also able to activate the jun/fos-transcription complex via an interaction with jun to upregulate expression of IL-6 (An et al. 2003a). Deregulation of the GSK-3β/β-catenin and jun/fos pathways induced the expression of inhibitor of DNA binding 1 (Id-1), a transcriptional repressor of CDKI, p16INK4A (Tang et al. 2003).

The potential of LANA-1 as a viral oncogene can be attributed to its ability to regulate the transcription of a number of genes that are important for cell cycle control, but to date, no mouse model for LANA-mediated oncogenesis has been described. However, the multiple functions of LANA-1 suggest that the protein is integral to the viral life cycle, and thus important in the pathogenesis of KSHV-driven tumours.

**KSHV-encoded transcription and cell cycle regulators:**

**vcyclin**

vcyclin, encoded by ORF72, is a cyclin homologue with 32% amino acid homology to cellular D-type cyclins (Cesarman...
et al. 1996; Li et al. 1997) and is present in all the known γ-2 herpesviruses. Like cellular D-type cyclins, vcyclin interacts with cdk4 and cdk6 to activate these kinases. Vcyclin-mediated deregulation of the cell cycle is attributed to its functional homology to cyclin complexes, including cyclin D/cdk4/6, cyclinE/cdk2 and cyclinA/cdk2. However, unlike the cellular cyclins, the kinase activity of the vcyclin/cdk complex is resistant to inhibitors p16INK4A, p21Cip and p27Kip (Swanton et al. 1997). Also, cyclin/cdk complexes are regulated by cyclin-activating kinases (CAKs), which phosphorylate conserved serine residues on cellular cdk subunits to activate their kinase activity. Although required for its full kinase activity, vcyclin/cdk6 can function independently of CAK phosphorylation (Kaldis et al. 2001). vcyclin/cdk6 independence from the requirement for phosphorylation along with its resistance to CDKIs suggests it is a constitutively active kinase.

The vcyclin/cdk6 complex has an extended range of substrates similar to the cyclin/cdk complexes involved in the G1/S phase transition of the cell cycle. Substrates include G0 D-type cyclin/cdk substrate, Rb (Godden-Kent et al. 1997), cyclinE/cdk2 substrate, p27Cip (Ellis et al. 1999; Mann et al. 1999) and cyclinA/cdk2 substrates, origin recognition complex-1 and Cdc6 (Laman et al. 2001).

The properties of vcyclin also extend to functions previously not ascribed to cyclins. For example, vcyclin interacts with signal transducer and activator of T cells 3 (STAT3) to inhibit its DNA binding and transcriptional activation, thus inhibiting the growth suppressive effects of oncostatin M signalling (Lundquist et al. 2003). Also, unique to vcyclin/cdk6 is its phosphorylation of cellular bcl-2, leading to inactivation of its anti-apoptotic properties (Ojala et al. 2000).

The ability of vcyclin to function like the G1/S phase cyclins with an unregulated kinase activity results in the independence of the cell cycle on growth signalling and the loss of normal cell cycle checkpoints, allowing cells to exit from quiescence and re-enter cell cycle (Swanton et al. 1997; Child & Mann 2001). The oncogenic potential of vcyclin has been tested in a transgenic mouse model where expression was shown to induce lymphomas after a long latency, suggesting somatic mutation are important to vcyclin-mediated lymphomagenesis (Verschuren et al. 2004). Moreover, when vcyclin transgenic mice were crossed to p53 null mice, these progeny developed lymphoma earlier suggesting apoptosis was a barrier to oncogenesis when vcyclin was overexpressed (Ojala et al. 1999; Verschuren et al. 2002). In the context of KSHV infection, vcyclin is likely to co-operate with the other latent expressed genes of KSHV, like LANA-1, that inactivate the p53 pathways, in order to potentiate KSHV-induced oncogenesis.

Conclusion

KSHV infection in tandem with other factors, such as secondary immunodeficiencies (age, iatrogenic immunosuppression and AIDS), are responsible for the development of KS, PEL and MCD. KSHV-induced tumourigenesis is probably attributed to expression and co-operation between KSHV lytic and latent proteins. KSHV encodes genes both unique to the γ-2 herpesviruses and homologous to cellular genes that are capable of activating growth-signalling pathways and deregulating the cell cycle, which benefit the viral life cycle and contribute to oncogenic transformation. These genes are therefore candidate viral oncogenes, constitutively activating growth-signalling pathways through their similarity to growth factors, mitogen-signalling receptors, signal transduction proteins, transcription regulatory factors and cell cycle proteins. Studying these signalling pathways has provided clues important to the oncogenic transformation of endothelial cells and B-cells, and may therefore yield new insight into therapeutic targets for the treatment of KSHV-associated tumours. These cellular pathways are also often mutated in nonvirally associated tumours. The KSHV viral oncogenes with homology to cellular genes provide uniquely evolved models for studying the biology of their proto-oncogenic cellular counterparts and the viral and cellular interactions, which result in cancer.

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