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Molecular virology of Kaposi's sarcoma-associated herpesvirus

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Kaposi's sarcoma-associated herpesvirus (KSHV), the most recently discovered human tumour virus, is the causative agent of Kaposi's sarcoma, primary effusion lymphoma and some forms of Castleman's disease. KSHV is a rhadinovirus, and like other rhadinoviruses, it has an extensive array of regulatory genes obtained from the host cell genome. These pirated KSHV proteins include homologues to cellular CD21, three different β-chemokines, IL-6, BCL-2, several different interferon regulatory factor homologues, Fas-ligand ICE inhibitory protein (FLIP), cyclin D and a G-protein-coupled receptor, as well as DNA synthetic enzymes including thymidylate synthase, dihydrofolate reductase, DNA polymerase, thymidine kinase and ribonucleotide reductases. Despite marked differences between KSHV and Epstein–Barr virus, both viruses target many of the same cellular pathways, but use different strategies to achieve the same effects. KSHV proteins have been identified which inhibit cell-cycle regulation checkpoints, apoptosis control mechanisms and the immune response regulatory machinery. Inhibition of these cellular regulatory networks appears to be a defensive means of allowing the virus to escape from innate antiviral immune responses. However, due to the overlapping nature of innate immune and tumour-suppressor pathways, inhibition of these regulatory networks can lead to unregulated cell proliferation and may contribute to virus-induced tumorigenesis.

Keywords: Kaposi's sarcoma; tumour virus; rhadinovirus; lymphoma; Castleman's disease; molecular piracy

1. INTRODUCTION

The human γ-herpesviruses, Kaposi's sarcoma (KS)-associated herpesvirus (KSHV or HHV-8) and Epstein–Barr virus (EBV or HHV-4), are extraordinary molecular tools for uncovering fundamental pathways in cell biology and tumorigenesis. As large double-stranded viruses, both KSHV and EBV possess proteins affecting multiple regulatory pathways in the cell.

KSHV is a γ_2-herpesvirus (genus *Rhadinovirus*) having sequence similarity with other rhadinoviruses including the prototype virus, *Herpesvirus saimiri* (HVS) (Albrecht *et al.* 1992; Fickenscher & Fleckenstein, this issue). KSHV was first discovered in 1994 through isolation of DNA fragments of open reading frames (ORFs) *26* and *75* from a KS lesion using representational difference analysis (Chang *et al.* 1994). The search for an infectious cause for KS was initiated from the unique epidemiology of these tumours in persons with and without AIDS (for background, see Beral *et al.* 1990). Subsequent epidemiological studies have unambiguously demonstrated that KSHV is the causative agent for all forms of KS and is present in virtually all pathologically confirmed KS specimens (for reviews, see Sarid *et al.* 1999*a*; Schulz *et al.* 1998; Schulz 2000; Boshoff & Weiss 1998 and this issue).

In addition to KS tumours, KSHV is found in B-cell primary effusion lymphomas (PELs) or body-cavity-based lymphomas (Cesarman *et al.* 1995*a*) and in some forms of multicentric Castleman's disease (CD), a B-cell lymphoproliferative disorder (Soulier *et al.* 1995). To date, only cell lines derived from PELs readily maintain the virus and can be cultured in the laboratory. These PEL cell lines are frequently coinfected with EBV (Cesarman *et al.* 1995*b*) but cell lines containing only KSHV at 20–150 virus copies per cell have been generated (Gao *et al.* 1996*b*; Renne *et al.* 1996; Arvanitakis *et al.* 1996). Asymptomatically infected individuals appear to harbour KSHV in $CD19^+$ B lymphocytes (Ambroziak *et al.* 1995), although KSHV clearly can also infect spindle cells in KS lesions presumed to be derived from endothelial progenitors.

Diseases caused by KSHV are highly dependent on the host's underlying immune status. Current evidence suggests that most individuals infected with KSHV remain asymptomatic unless they acquire coexistent immunodeficiency (e.g. through chemotherapy or human immunodeficiency virus (HIV) infection). In this setting, KSHV-infected individuals have a very high risk of developing KS and other KSHV-associated diseases. There is little current support for the concept that HIV or its gene products (e.g. tat protein) directly promote KS tumour growth, in particular since HIV is frequently not present in appreciable quantities in KS tumours (Mahoney *et al.* 1991). High rates of KS occurring among AIDS patients are probably due to severe cellular immunodeficiency from HIV infection in persons who share risk factors for infection with both HIV and KSHV. The reasons for KS occurring in elderly, HIV-negative

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Table 1. *Correspondence between KSHV and EBV regulatory genes*
(From Moore & Chang (2001).)

KSHV genes	effect	cell protein induced by EBV	responsible EBV proteins
ORF 4 (vCBP)	complement inactivation?	CD21/CR2	EBNA-2
ORF K2 (vIL-6)	growth factor	IL-6	gp350/220 and LMP-1
ORF 16 (vBCL-2)	anti-apoptosis	BCL-2	LMP-1
ORF K9 (vIRF)	IFN inhibition, cMYC activation CBP/p300 binding	cMYC	EBNA-2
ORF 72 (vCYC)	cell-cycle control	cyclin D2	EBNA-2 and EBNA-LP
ORF 74 (vGCR)	cellular proliferation	EBI-1	EBV induced
ORF K1 and *K15* (LAMP)	immunoreceptor signalling	—	LMP-1, LMP-2
ORF 50 (ART)	lytic transactivator	—	BRLF (Rta)
ORF K8 (Kb-ZIP)	lytic transactivator?	—	BZLF (Zta, ZEBRA)
ORF 73 (LANA)	*ori-P*-binding protein	—	EBNA-1

individuals in endemic areas is poorly understood but possible explanations include high infection rates in specific geographical regions, age-dependent declines in cellular immunity and HLA haplotype-dependent immune surveillance.

The genomic organization of KSHV is broadly similar to other rhadinoviruses. KSHV is an approximately 165 kb double-stranded DNA virus whose genome consists of a long unique region (LUR) *ca.* 145 kb in length. Flanking the LUR are variable numbers of 801 bp direct terminal repeat (TR) units having a high G + C content (85%) (Neipel *et al.* 1997*b*; Russo *et al.* 1996). The TR region has not been found to encode any ORFs but does appear to possess the *ori-P* recognition sequence allowing tethering of KSHV episomes to chromosomal DNA by the latency-associated nuclear antigen (LANA-1) protein during mitosis (Ballestas *et al.* 1999; Cotter & Robertson 1999). As a latent episome, the virus is circularized through the TR region. When the virus genome undergoes lytic replication and packaging into virions through a rolling-circle mechanism, it is replicated as a linear molecule capped on both sides by varying numbers of TR units.

2. EPSTEIN–BARR VIRUS AND KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS: A COMPARISON

Despite gross differences between KSHV and EBV genomes, the two viruses share a remarkable degree of biological similarity. Both are γ-herpesviruses and reside in a B-cell reservoir in their natural host. Each virus also demonstrates tropism for lymphoid-cell lineages. Both may have to infect and cross epithelial cells during initial infection of mucosal surfaces prior to B-cell entry. It is therefore not surprising that these viruses face similar cellular environments and obstacles. KSHV and EBV have striking functional similarities but have evolved entirely different mechanisms for maintaining their viral episomes in the hostile environment of the host cell (table 1).

(a) *Lytic virus replication genes*

A cursory examination of the KSHV genome for regional similarity with EBV (figure 1) reveals that both viruses maintain the basic herpesvirus structural architecture that has proven so successful for this family of viruses. As expected, encapsidated KSHV virions have a similar ultrastructural appearance to other herpesviruses (Renne *et al.* 1996; Orenstein *et al.* 1997). Most of the major structural and lytic-cycle regulatory genes found in distantly related herpesviruses are recognizable in both KSHV and EBV (Neipel *et al.* 1997*b*; Russo *et al.* 1996), suggesting that the fundamental processes of virus assembly, egress and re-entry are likely to be very similar.

KSHV genes are named from left to right after HVS genes in a standard representation, which is reversed relative to the standard EBV representation. Genes not recognizably represented in the HVS genome are given a 'K' designation. The KSHV genome (figure 1) is divided into blocks of highly conserved structural and lytic replication genes interspersed between blocks showing little or no similarity to other herpesviral genes. Transcriptional mapping studies performed in naturally infected PEL cell lines show that many of the non-conserved KSHV genes respond to phorbol ester treatment in a similar manner (figure 1). This suggests that conserved and non-conserved genes tend to be physically clustered together on the genome by transcriptional regulatory patterns (Sarid *et al.* 1998).

While all herpesviruses encode DNA synthesis genes, KSHV possesses a more extensive array of homologues of cellular DNA synthetic enzymes than EBV. In addition to the DNA polymerase (*ORF 9*), thymidine kinase (*ORF 21*), and ribonucleotide reductase (*ORF 60* and *ORF 61*) genes, KSHV possesses a thymidylate synthase (*ORF 70*), a dihydrofolate reductase (*ORF 2*) and FGARAT (*ORF 75*, N-formylglycinamide ribotide amidotransferase). The cellular versions of many of these genes are regulated during G1 and S phases by the E2F transcriptional factor indicating that KSHV homologues may either supplement these regulated cellular genes or extend S-phase DNA synthesis into other phases of the cell cycle during lytic replication. These DNA synthesis genes are potential targets for antiviral drugs that may inhibit lytic replication (Cannon *et al.* 1999*a*; Cinquina *et al.* 2000).

In addition to DNA synthesis enzyme genes, differences exist between EBV and KSHV in lytic transcriptional activators. Homologues of the EBV BZLF1 (Zta) and

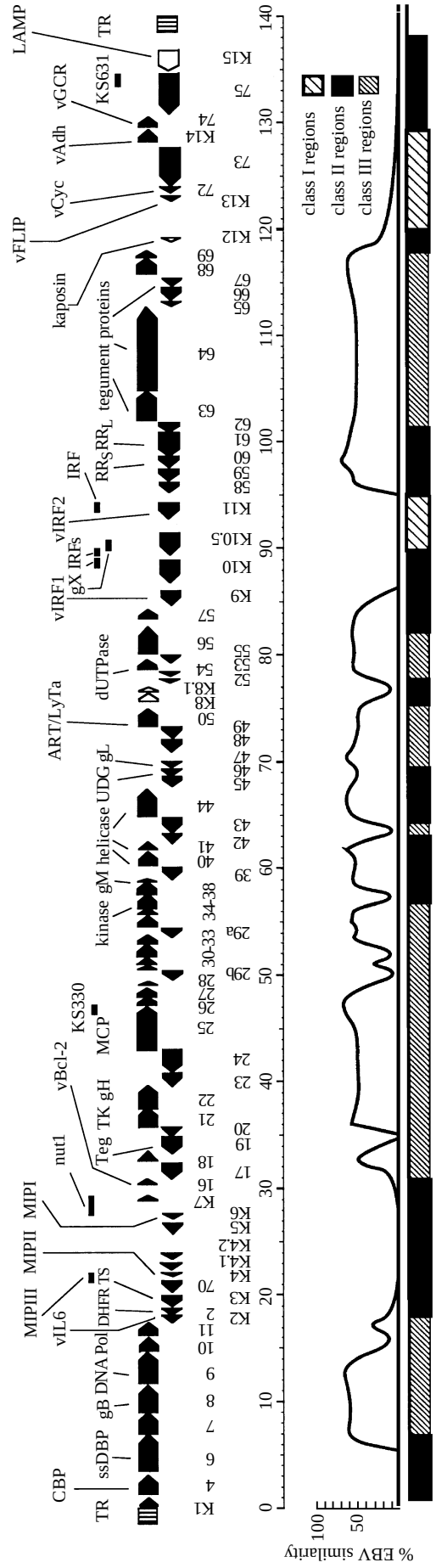

Figure 1. A representation of the KSHV genome showing the major ORFs in the LUR. Below the genomic representation is a chart figure showing the percentage amino-acid identity represented for each KSHV ORF and the corresponding ORFs in EBV. This graph shows that highly conserved genes, mainly encoding structural and virion replication proteins, tend to cluster together while intervening areas are rich in latency genes that have little or no similarity between the two viruses. Clusters of KSHV genes also tend to be regulated similarly with distinct clusters of genes having expression patterns in PEL cells falling into either class I (constitutive), class II (constitutive-TPA inducible) or class III (only expressed after TPA induction) patterns. Reproduced with permission from Moore & Chang (2001).

BRLF1 (Rta) transactivators are encoded by KSHV *ORF K8* (Kb-ZIP) and *ORF 50* (ART, activator of protein of replication and transcription), respectively (Sun *et al.* 1998; Lukac *et al.* 1998, 1999; Lin *et al.* 1999; Gruffat *et al.* 1999). Both KSHV transactivators are spliced gene products. In contrast to EBV, ART is the major viral lytic-replication transactivator and induces expression of Kb-ZIP as an immediate-early gene product (Sun *et al.* 1999). ART activates its own promoter, resulting in rapid amplification of the viral transcriptional cascade through a positive feedback mechanism (Gradoville *et al.* 2000).

KSHV lytic gene transcription is controlled through transcriptional cascades by ART. Most transcriptional studies of KSHV are based on PEL cell lines that are inducible into productive virus replication using phorbol esters or histone deacetylase inhibitors (e.g. butyrate). It is clear now, however, that constitutive KSHV gene transcription is tissue dependent and that the act of establishing cell lines *in vitro* results in dysregulation of some genes (Moore *et al.* 1996*a*; Parravicini *et al.* 1997*a*, 2000; Staskus *et al.* 1999). Responsiveness of KSHV genes to chemical inducing agents, like phorbol esters, is not an optimal means for gauging whether or not a given gene is likely to be expressed during latency or lytic replication, since some viral promoters are directly activated by phorbols. For these reasons, gene expression patterns must be determined directly *in situ* in tissues, as well as in cell culture models. Initial efforts have been made to dissect lytic transcriptional cascades involved in early and late viral gene transcription (Chang & Ganem 2000). These studies suggest that late viral gene transcription is dependent on KSHV polymerase activity.

(b) *Homologues of cell-regulatory genes*

Prominent differences between KSHV and EBV are found among genes responsible for modifying the cellular environment during viral latency. None of the major EBV genes involved in cell transformation or latency is directly represented in the KSHV genome. Nevertheless, monoclonality of the viral genome in PEL cells and in some KS lesions (other KS lesions appear to have a polyclonal origin) suggests that KSHV can contribute directly to oncogenic processes, as can EBV in other contexts (Russo *et al.* 1996; Judde *et al.* 2000). KSHV is also able to induce polyclonal cell proliferation as is seen in CD (Dupin *et al.* 1999; Boshoff & Weiss, this issue).

The most striking feature of KSHV is its extensive use of molecular piracy to capture cellular-regulatory genes. Most of these genes do not have introns and the mechanism by which they became incorporated from cellular cDNA is unknown (Neipel *et al.* 1998). Viral proteins encoded by these genes (in reading frames in parentheses) include homologues of cellular complement receptor 2 (*ORF 4*), three CC family chemokines (*ORF K4*, *ORF K4.1* and *ORF K6*), an interleukin (IL)-6 homologue (*ORF K2*), a BCL-2 homologue (*ORF 16*), at least three descendants of an ancestral interferon (IFN)-regulatory transcription factor (*ORF K9*, *ORF K10.5*, and a yet to be designated ORF between *ORF K11* and *ORF 58*), a D-type cyclin (*ORF 72*), a Fas-ligand interleukin converting enzyme (FLICE)-like caspase inhibitor (FLIP, encoded by *ORF K13*), a CXC-like chemokine receptor G-protein-coupled receptor (GPCR) homologue (*ORF 74*) and a transmembrane-spanning adhesion molecule (*ORF K14*). Strikingly, most of the proteins encoded by these captured genes are functionally similar to cellular proteins induced by EBV infection (table 1). Some of these genes are expressed constitutively, while others are either expressed in various phases of latent infection or are expressed primarily during lytic replication.

(c) *KSHV latency gene regulation*

Although controversy exists over the transcriptional classification of some genes, classification of constitutively expressed KSHV genes as latent genes is widely accepted. Several methods have been used to globally map viral gene transcription (Zhu *et al.* 1999; Sun *et al.* 1999; Sarid *et al.* 1998; Renne *et al.* 1996; Zhong *et al.* 1996). These studies are broadly similar in showing that most genes are transcriptionally silent in the absence of chemical inducing agents. Since most PEL cell lines have a low but detectable level of spontaneous virion production, care needs to be taken to ensure that gene transcription levels do not reflect a mixture of latent and lytic cell populations.

At the far right end of the genome, a cluster of three genes, on two polycistronic transcripts (LT1 and LT2), is constitutively expressed in PEL cells (Dittmer *et al.* 1998; Sarid *et al.* 1999*b*; Talbot *et al.* 1999). The LT1 transcript encodes the viral (v) FLIP (vFLIP), cyclin (vCYC) and LANA-1 (*ORF 73*) proteins, while the LT2 transcript originates from the same cell-cycle-regulated promoter but splices out *ORF 73*. Immunohistochemical staining demonstrates that LANA-1 protein is present in nearly all infected cells from tumours and cell cultures, suggesting that it, like its EBV analogue Epstein–Barr nuclear antigen (EBNA)-1, is a universal marker for KSHV infection (Dupin *et al.* 1999; Parravicini *et al.* 2000). A fourth gene, *K10.5*, encoding LANA-2, has recently been discovered that has a similar expression pattern in infected haematopoietic cells (including PELs and CD) but not KS tumours (Rivas *et al.* 2001; Y. Mori and F. Neipel, unpublished data). While vCYC transcription has generally been found to be constitutive in PEL cell lines, protein expression has been reported to be variable (Carbone *et al.* 2000; Platt *et al.* 2000).

Understanding the pathogenic consequences of expression of any given KSHV gene for a KSHV-related disease requires direct examination of the diseased tissues. Two groups have initiated protein expression surveys (Katano *et al.* 2000; Parravicini *et al.* 2000) using antibodies directed against KSHV proteins. Proteins examined included LANA-1, viral IFN-regulatory factor 1 (vIRF1), vIL-6, processivity factor 8 (PF-8) (*ORF 59*), and proteins encoded by ORFs *26*, *K8*, *K8.1*, *K10*, *K11* and *65*. Similar results were obtained by both groups in that KSHV gene expression is tightly regulated in most tumours and different protein expression patterns exist for different cell types.

3. KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS PROTEINS AFFECTING CELL-REGULATORY PATHWAYS

Despite the short time elapsed since the discovery of KSHV, rapid progress has been made in understanding

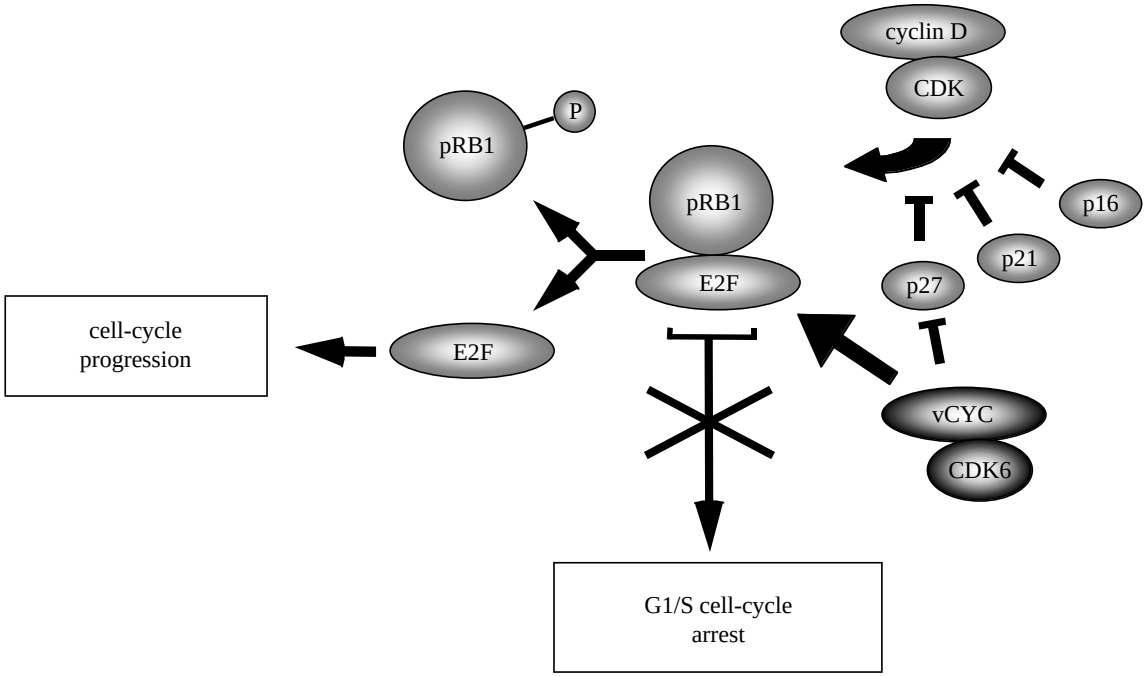

Figure 2. Viral CYC inhibition of pRB1 through recruitment of CDKs. The pRB1 protein serves as a major checkpoint regulator for allowing the cell to progress through the G1 and S checkpoint by binding to and inhibiting E2F transcription factors. D-type cyclins couple with CDK4 or 6 to phosphorylate and inactivate pRB1, thus allowing cell-cycle progression. CDKIs, such as p16, p21 and p27 phosphorylate the cyclin-CDK complex to inhibit this process. Viral CYC can induce cell proliferation and pRB1 phosphorylation even in the presence of strong CDKI activity since vCYC is resistant to inhibition by CDKIs.

the functions of many KSHV proteins. This has largely been due to the ability of investigators to predict functions of viral proteins based on their homology to known cellular proteins.

Single-gene studies have shown that KSHV possesses a complex molecular capacity to regulate cell proliferation. Transformation of rodent cell lines such as NIH3T3 or Rat-1 cells by exogenously expressed viral genes is a useful means to assay the effects of viral proteins on cell proliferation or apoptosis. There are a surprisingly large number of KSHV genes that transform rodent cell lines, including KSHV-immunoreceptor signalling protein (*ORF K1*), vIL-6 (*ORF K9*), kaposin (a protein encoded by one of multiple *ORF 12* transcripts), vIRF1 (*ORF K9*), and vGPCR (*ORF 74*). Additional KSHV proteins assumed to influence cell proliferation or apoptosis include LANA-1 and LANA-2 (*ORF 73* and *ORF K10.5*, respectively), vCYC (*ORF 72*) and vBCL-2 (*ORF 16*). A major emphasis of current research is to determine which genes are responsible for KSHV-driven tumour cell growth.

It is certain that additional novel cell-regulatory functions will be described for the KSHV proteins as individual viral genes are examined and new cellular genes and pathways are described. A great deal, however, is already known about the KSHV-regulatory genes that allows us to begin to piece together their functions in the infected cell. The regulatory proteins can broadly be classed into cell-cycle regulators, apoptosis inhibitors, proteins that mimic B-cell receptor (BCR) activity, immunomodulatory proteins and cytokines, and cytokine receptors. Most if not all of these proteins are active in at least some of the disorders caused by KSHV infection.

(a) *Cell-cycle regulators*

The prominent example of a gene that has potential to regulate the cell cycle is vCYC (*ORF 72*). This protein most closely resembles a D-type cyclin, especially in the cyclin box domain responsible for interaction with cyclin-dependent kinases (CDKs) (Cesarman *et al.* 1996). Cellular cyclins are the regulatory component of CDKs, which are serine-threonine kinases active in phosphorylation of cell-cycle regulatory components (for a review, see Mittnacht & Boshoff 2000). Cyclins provide the substrate specificity for CDK kinase activity and, hence, their cell-cycle-dependent synthesis and degradation is a key regulator for cell progression through the cell cycle. The D-type cyclins are expressed in G1 and S phases and primarily target CDKs to retinoblastoma protein (pRB1). Phosphorylation of pRB1 blocks its repressor activity on transcription factors such as E2F, which regulate DNA synthesis. By inhibiting the pRB1 gatekeeper, D cyclins initiate transit of the cell cycle from G1 to S and promote transcription of DNA synthesis enzyme genes.

Viral CYC acts in a similar fashion to the cellular cyclins (figure 2). It partners primarily with CDK6 and to a lesser extent with CDK4 and CDK5 (Li *et al.* 1997; Godden-Kent *et al.* 1997). This interaction results in pRB1 hyperphosphorylation, which can overcome cell senescence caused by pRB1 overexpression (Chang *et al.* 1996). Intriguingly, vCYC also directs phosphorylation of histone H1, which is not a normal substrate of D-type cyclins, but is a more characteristic target for other cyclin-CDKs such as cyclin A-CDK2. This suggests the possibility that vCYC may regulate other cell-cycle phases in addition to the G1 and S checkpoint.

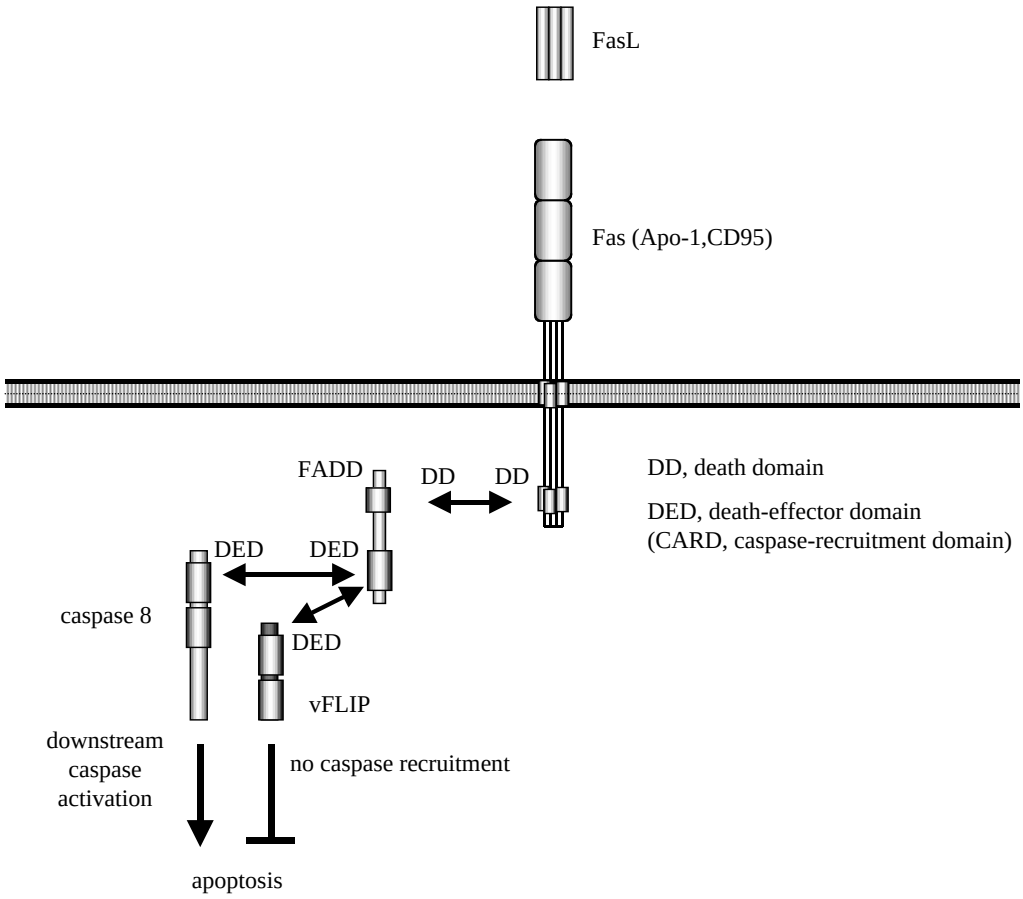

Figure 3. Mechanism for vFLIP inhibition of Fas-mediated apoptosis. After Fas ligand (FasL) activation of the Fas membrane receptor, multimerization of Fas induces aggregation with the Fas-adaptor protein (FADD) containing unique death domains, and in turn aggregation and activation of caspase 8 to form the DISC. Interaction between FADD and caspase 8 takes place through a second conserved motif (DED). Viral FLIP acts as a dominant-negative inhibitor of this process since it possesses two DED domains and binds FADD and/or caspase 8 without DISC formation.

Because of their critical regulatory role in the cell, cyclin-CDK complexes are in turn tightly regulated by cellular proteins called CDK inhibitors (CDKIs) (Swanton *et al.* 1999). Viral CYC, however, can escape these normal cell-regulatory circuits controlling cyclin function. Swanton *et al.* (1997) showed that vCYC is resistant to inhibition by CDKIs, including p16, p21 and p27. Viral CYC also directs phosphorylation of p27 resulting in its degradation (Mann *et al.* 1999; Ellis *et al.* 1999). Despite this, PEL cells with high mitotic indices also have high p27 and vCYC levels, whereas cellular cyclin D1 is undetectable (Carbone *et al.* 2000). Marked variation of vCYC protein expression has been reported between PEL cell lines, and some evidence suggests that vCYC transcription may differ between different cell lines as well (Platt *et al.* 2000).

Another potential cell-cycle regulator is the viral antigen LANA-1 (*ORF 73*), which functionally resembles the T antigen of SV40 polyomavirus. LANA-1 acts as a molecular bridge between the viral episome and cellular chromosomes, ensuring equal segregation of virus into daughter cells (Ballestas *et al.* 1999; Cotter & Robertson 1999). This allows equal segregation of episomes between dividing daughter cells. More recently, Radkov and colleagues have found LANA-1 to directly interact with pRB1, resulting in E2F-responsive promoter activation and an antagonism of pRB1-mediated cell-cycle arrest similar to that of vCYC (S. Radkov, P. Kellam and C. Boshoff, unpublished data). Together with H-ras, LANA-1 induces primary rat embryo fibroblast transformation. Since LANA-1 is expressed constitutively in KSHV-infected cells, it is a leading viral candidate protein to contribute to KSHV-induced tumorigenesis.

(b) *Inhibition of apoptosis*

Cellular apoptosis is a continuous threat to successful establishment of a viral infection. Apoptosis can prematurely kill the infected cell before optimized virion egress, thus reducing the chances for successful transmission to a new cell or new host. Therefore, viruses, including KSHV, have evolved strategies to inhibit the multiple cellular apoptotic pathways. KSHV anti-apoptotic proteins are not all constitutively expressed at the same time and therefore may only inhibit apoptotic functions in specific cell types or during particular phases of the virus's natural life cycle.

The vBCL-2 encoded by *ORF 16* was the first KSHV protein to be investigated for its apoptosis-inhibitory properties (Sarid *et al.* 1997; Cheng *et al.* 1997). Like cellular members of this family, vBCL-2 contains conserved BH1 and BH2 domains. While conflicting evidence still exists regarding vBCL-2's ability to heterodimerize with cellular BCL-2/Bax family members, there is widespread agreement that BCL-2 is able to inhibit

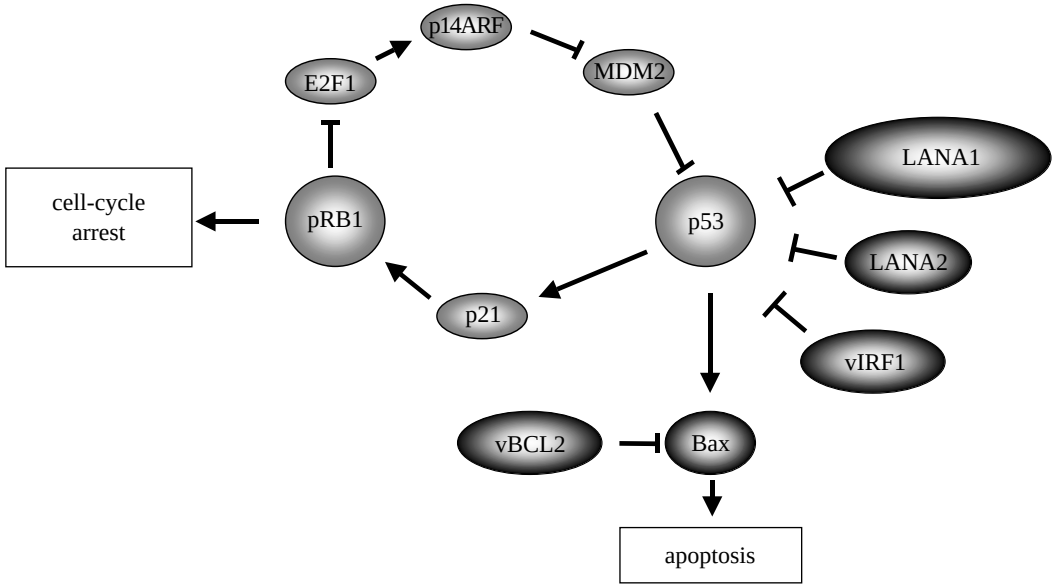

Figure 4. Known KSHV inhibitors of the p53 tumour-suppressor protein. Inappropriate activation of mitogenic pathways, for example inhibition of pRB1 by vCYC, generally activate p53 through a feedback mechanism involving the p14ARF (alternative reading frame) protein. LANA-1, LANA-2 and vIRF1 have been shown to directly inhibit p53 transcriptional activity and thus may block the effects of p53 activation. These proteins are expressed in different cell types and phases of the viral life cycle.

Bax-related apoptosis. Viral BCL-2, in contrast to cellular BCL-2, is able to prevent apoptosis due to vCYC–CDK6 overexpression, suggesting important differences in regulation of the two proteins (Ojala *et al.* 1999). KSHV BCL-2 is principally expressed in tissue culture cell lines after phorbol stimulation, presumably to inhibit apoptosis during lytic replication. It is not appreciably expressed in KS tumours.

The vFLIP (*ORF K13*, also designated *ORF 71*) belongs to the newly discovered class of FLIPs. The vFLIPs were first identified from KSHV and other rhadinoviruses, as well as from molluscum contagiosum virus, based on their possession of characteristic death effector domains (DEDs) (Bertin *et al.* 1997; Thome *et al.* 1997; Hu *et al.* 1997). Subsequently, cellular homologues of these proteins were discovered (Irmler *et al.* 1997). The vFLIP possesses a single DED domain, allowing it to bind to the death-inducing signalling complex (DISC) but preventing recruitment of caspase 8 (figure 3). The vFLIP can thus act as a dominant-negative inhibitor of Fas-tumour necrosis factor receptor (Fas-TNFR) signalling pathways. Independently of its FLICE-interactions, vFLIP also activates nuclear factor kappa B (NF-κB) signalling, a function analogous to that of EBV latent membrane protein 1 (LMP-1) (Hammarskjold & Simurda 1992), by activating upstream kinases that initiate degradation of the NF-κB inhibitor, IκB (Chaudhary *et al.* 1999).

Three different KSHV proteins directly target the pro-apoptotic p53 tumour-suppressor protein (figure 4). Overall, LANA-1 is an highly acidic protein, composed of a basic 330 residue N-terminus, followed by an internal acidic repeat region termed moi, composed of glu–asp–pro or glu–asp–gln repeat sequences and ending with a leucine zipper domain. This is followed by the C-terminal 190 amino acids that have an overall basic charge. The charge structure of this protein suggests that it may have strong internal attractions and indeed it runs on denaturing electrophoresis gels as a 220 kDa doublet, considerably higher than its predicted 150 kDa size (Gao *et al.* 1996*a*). LANA-1 is post-translationally modified by small ubiquitin-like modifier 1 (SUMO-1) and by phosphorylation, but it is not known in what ways this may change its function (R. Sarid, P. S. Moore and Y. Chang, unpublished data).

LANA-1 was first identified by serological means (Moore *et al.* 1996*b*) and remains one of the most important antigens for KSHV serological assays. LANA-1 expression was found to efficiently inhibit p53 activation of a promoter containing the multimerized p53 element (pG13) from p21, whereas a truncated mutant possessing only the first 440 amino acids had no activity (Friborg *et al.* 1999). When LANA-1 and p53 are overexpressed in p53-null cells, LANA-1 diminishes the apoptotic response generated by p53. It is not clear whether all, or only some, of the multifunctional p53 pathways leading to cell-cycle arrest or apoptosis are inhibited by p53. Nonetheless, this is an attractive candidate protein for contributing to KSHV-related neoplasia since it is universally expressed and it inhibits pRB1 as well as p53.

A functionally similar protein to LANA-1 is the newly described LANA-2 protein encoded by *ORF 10.5* (Rivas 2001; Y. Mori and F. Neipel, unpublished data). This ORF belongs to a group of genes and sequence motifs possessing varying degrees of homology to the IRF family of transcription factors. LANA-2 is translated from a spliced transcript and thus this gene was not identified by sequence analysis in the original descriptions of the genome. It contains a small region with homology to the human IRF4 interaction domain. LANA-2 is expressed in cells and cell lines (i.e. PEL, PEL cell lines and CD tumours) of haematopoietic origin but not in KS tumours. Like LANA-1, LANA-2 is a latent protein whose expression is not appreciably induced by phorbols esters or repressed by DNA polymerase inhibitors. LANA-2 inhibits

p53-induced transcription and apoptosis although it is unclear whether this is due to direct binding to p53. These findings suggest that LANA-2 may act in lymphocyte-specific settings to abrogate p53 activation during viral latency. While it contains an IRF family motif and demonstrates sequence similarity to KSHV vIRF1, LANA-2 does not appear to inhibit IFN signalling.

A third KSHV protein known to inhibit p53 function is the vIRF1 protein encoded by *ORF K9*. Like LANA-1 and LANA-2, vIRF1 also binds p53 and inhibits p53-induced apoptosis (S. Jayachandra, Y. Chang and P. S. Moore, unpublished data). Viral IRF1 is a multifunctional viral protein that inhibits IFN-induced gene expression and activates *c-myc* expression by binding to transcriptional coadaptors (see § 3(d), figure 5). Studies are ongoing to determine if vIRF1 binding to p53 is direct or bridged by binding to coadaptors that also bind p53 (Lill *et al.* 1997; Avantaggiati *et al.* 1997). Viral IRF1 protein is expressed in hyperplastic CD tissues but it is not expressed in PELs or KS tumours (dysregulation of gene expression occurs in PEL tissue culture cells, however (Parravicini *et al.* 2000)).

The reasons for this extraordinary redundancy in p53-inhibition by KSHV proteins are unknown. It can be speculated that p53 must be inactivated at different times or in different tissues by these viral proteins, each of which must have specialized regulatory or expression patterns. This redundancy reinforces the critical nature of p53 as a regulator of viral replication. It appears that inhibition of p53 is a near-universal feature of DNA tumour viruses (Moore & Chang 1998).

(c) *KSHV immunomodulatory proteins*

Escape from both innate and adaptive immune surveillance is also critical for viral survival and propagation. Innate antiviral immunity involves several pathways, including IFN and Fas signalling pathways, which act in a non-specific manner to reduce the efficiency of viral replication. Adaptive immunity involves generation of a specific cytotoxic lymphocyte (CTL) response against specific viral peptide epitopes that have been processed and presented by the cellular major histocompatibility complex (MHC) antigens. Innate immunity is a blunt hammer that can be immediately used against viral infection, giving time for the adaptive immune scalpel to be prepared.

Several proteins encoded by KSHV target intracellular immune surveillance pathways in unique ways. In this section, we focus on KSHV proteins affecting IFN signalling and MHC antigen presentation. Other KSHV proteins also act on immune pathways (KSHV cytokines and KSHV proteins mimicking BCR functions) but will be dealt with in § 3(e) and (f). The KSHV anti-immune functions are enlightening since several proteins (vIRF-1, ORF K1 protein and vIL-6) dually inhibit immune surveillance and promote cell transformation, illustrating the interconnectedness of immune and tumour-suppressor signalling pathways.

(d) *vIRF1 and IFN signalling pathways*

The IRF family of transcription factors is involved in regulating gene expression in response to IFN signalling. They have an N-terminal DNA-binding motif and a C-terminal transactivator or repressor region. The vIRF1 protein encoded by *ORF K9* was the first described viral member of the IRF family (Moore *et al.* 1996*a*). It is a 449 amino-acid protein possessing a central region with similarity to the DNA-binding domain motif and weaker similarity in the transactivator region. Unlike other IRF members, it possesses a leading 89 amino-acid N-terminus segment without known homology to cellular proteins. Despite possessing a region similar to the IRF DNA-binding domain, vIRF1 does not directly bind DNA. Viral IRF-1 inhibits transcription of IFN-responsive gene expression, downregulates p21 and prevents IFN-induced growth arrest (Gao *et al.* 1997; Li *et al.* 1998; Zimring *et al.* 1998; Flowers *et al.* 1998). While vIRF1 generally acts as a transcriptional repressor, evidence exists that it can act as a transcriptional activator at specific viral and cellular promoters (Roan *et al.* 1999; Jayachandra *et al.* 1999; Li *et al.* 1998). Viral IRF1 expression fully transforms NIH3T3 cells into cancerous cells (Gao *et al.* 1997), similar to the cellular IRF-2 protein (Harada *et al.* 1993).

IFNs can be divided into the two major classes: class I, which includes IFN-α and -β, and class II, which includes IFN-γ. Class I and class II IFNs have different receptors and signal transduction pathways responsible for promoting an antiviral state in the cell (for a review, see Taniguchi *et al.* 1997). A simplified diagram of the class II (IFN-γ) pathway is shown in figure 5. IFN-γ binds to its receptor causing Janus kinase phosphorylation, which in turn results in activation and homodimerization of the signalling transduction and transcription (STAT)-1 factor. Activated STAT-1 homodimers migrate to the nucleus where they act as transcription factors by binding γ-activated sequence (GAS) elements in IFN-responsive promoters. The second pathway for IFN signalling is activated when class I IFN binds to its receptors, activating Jak-Tyk kinases to phosphorylate STAT-1, STAT-2 and p48. These three proteins form a trimeric complex, IFN-stimulated gene factor 3 (ISGF3), which, in an analogous fashion to STAT-1, migrates to the nucleus to activate genes containing IFN-stimulated response (ISRE) elements.

Crosstalk between the two pathways appears to occur through at least two mechanisms (Taniguchi *et al.* 1997). Spreading of the class II (IFN-γ) response into the class I (IFN-α and -β)-regulated pathways occurs through induction of the IRF1 protein, which is upregulated by class II signalling. IRF1 is similar to ISGF3 since it activates many genes containing ISRE sequences (Miyamoto *et al.* 1988). Therefore, IFN-γ signalling leads to secondary activation of promoters containing ISRE as well as GAS elements. Spreading of class I IFN signalling into class II pathways also seems to occur since STAT-1 activation by class I IFNs leads to STAT-1 homodimerization as well as activation of ISGF3 (Taniguchi *et al.* 1997). The exact patterns of gene activation from class I and II IFN signalling appear to be further complicated by specific modifiers, including other IRF family members, that alter patterns of gene expression in different tissue types and cell activation states (Nelson *et al.* 1993; Weisz *et al.* 1994).

Viral IRF1 inhibits these signalling pathways by binding to transcriptional coadaptors. Transcription coadaptors are proteins that link specific transcription factors to the

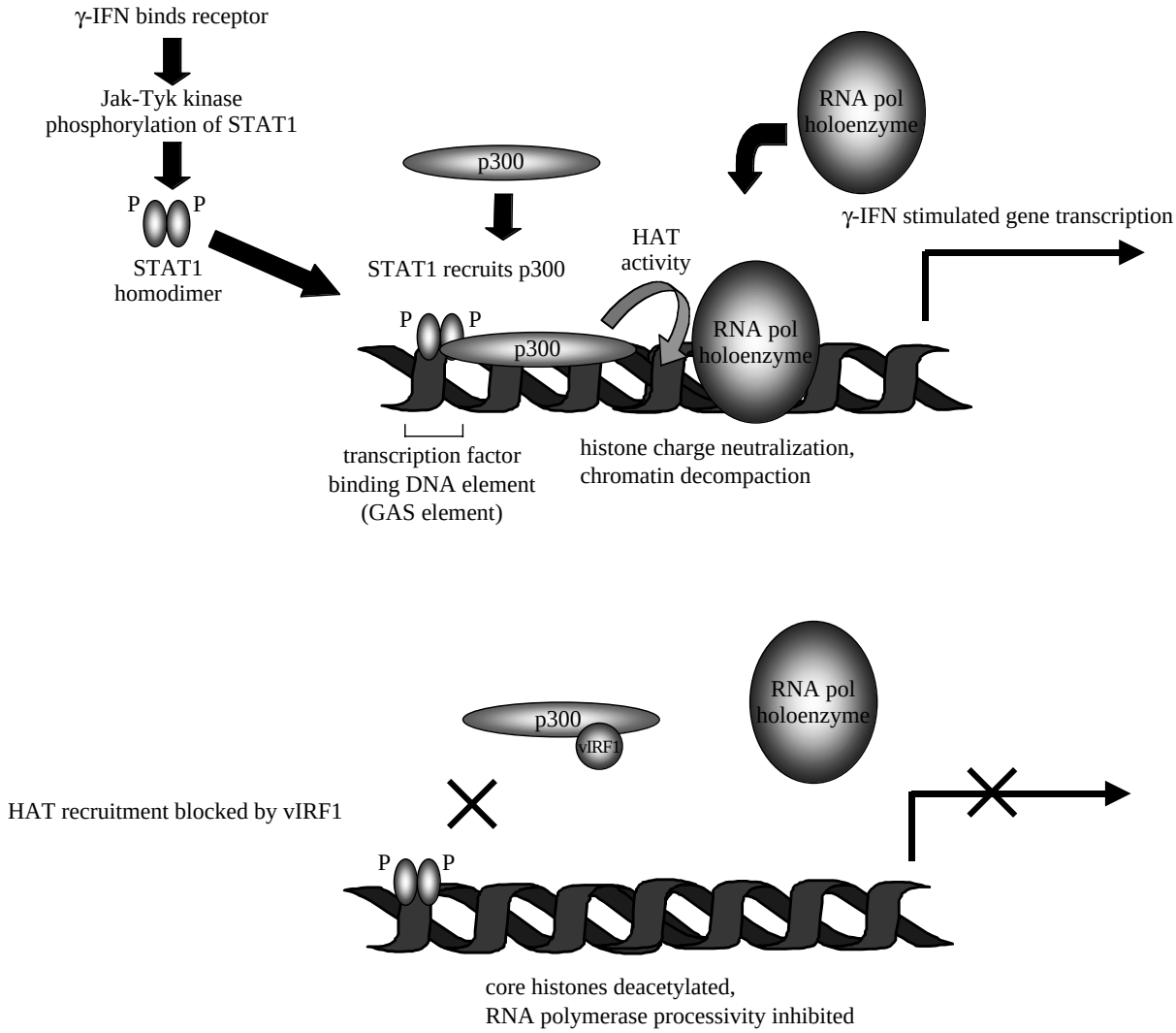

Figure 5. Model for vIRF1 inhibition of IFN-γ signal transduction via sequestration of transcriptional coadaptors. As shown here, IFN-γ activates STAT-1, which homodimerizes and travels to the nucleus to act as a transcription factor for promoters containing GAS elements. This requires recruitment by STAT-1 of HAT coadaptors, such as CBP or p300, to acetylate and neutralize the basic charge on core histones to allow chromatin relaxation. vIRF1 binds to p300 and CBP and prevents these coadaptors from being recruited to STAT-1, thus inhibiting transcription. A similar pathway exists for class I IFN-α and -β signalling, which acts on promoters containing ISREs.

non-specific basal transcriptional machinery and RNA polymerase holoenzyme complex to initiate transcription. One function of coadaptors is acetylation of core histones, which relaxes condensed chromatin and serves as a recruitment signal for other transcription molecules such as transcription activating factor II250 (Jacobson *et al.* 2000). The most widely studied histone acetyltransferase (HAT) coadaptors include the related proteins, p300 and cyclic AMP-response element (CREB)-binding protein (CBP), as well as the p300 and CBP-associated factor (for a review, see Goodman & Smolik 2000). Viral oncoproteins can efficiently inhibit IFN transcription by binding HAT coadaptors, sequestering them away from STAT1 or ISGF3 complexes in the IFN signalling pathways (figure 5). Known viral inhibitors of HAT coadaptors include EBV EBNA-2 (Jayachandra *et al.* 1999; Wang *et al.* 2000), adenovirus E1A (Yang *et al.* 1996; Bhattacharya *et al.* 1996; Zhang *et al.* 1996), SV40 T antigen (Ludlow & Skuse 1995), human T-cell leukaemia virus type 1 Tax (Kwok *et al.* 1996) and papillomavirus E6 proteins (Patel *et al.* 1999). Viral IRF1 binds to CH2 and CH3 domains of p300 and CBP (Burysek *et al.* 1999; Jayachandra *et al.* 1999) and, when expressed in cells, markedly inhibits both IFN class I (Gao *et al.* 1997) and class II (Zimring *et al.* 1998) IFN-promoter activation.

IFN generally induces cell-cycle arrest through the downregulation of *c-myc* (Jonak & Knight 1984) and upregulation of the CDK p21 promoter (Tanaka *et al.* 1996; Hobeika *et al.* 1997; Sangfelt *et al.* 1997). This is an effective strategy to limit viral replication since it prevents viral, as well as cellular, nucleic acid replication and primes the infected cell for apoptotic death. Viral IRF-1 inhibition of IFN signalling may prevent these effects as well as the activation of other genes associated with an antiviral state.

While vIRF1 represses transcription of most IFN-regulated promoters, such as the p21 promoter, it can activate promoters that are repressed during IFN signalling, such as the *c-myc* promoter. The way this occurs reveals insights into how tumour virus oncogenes can modify

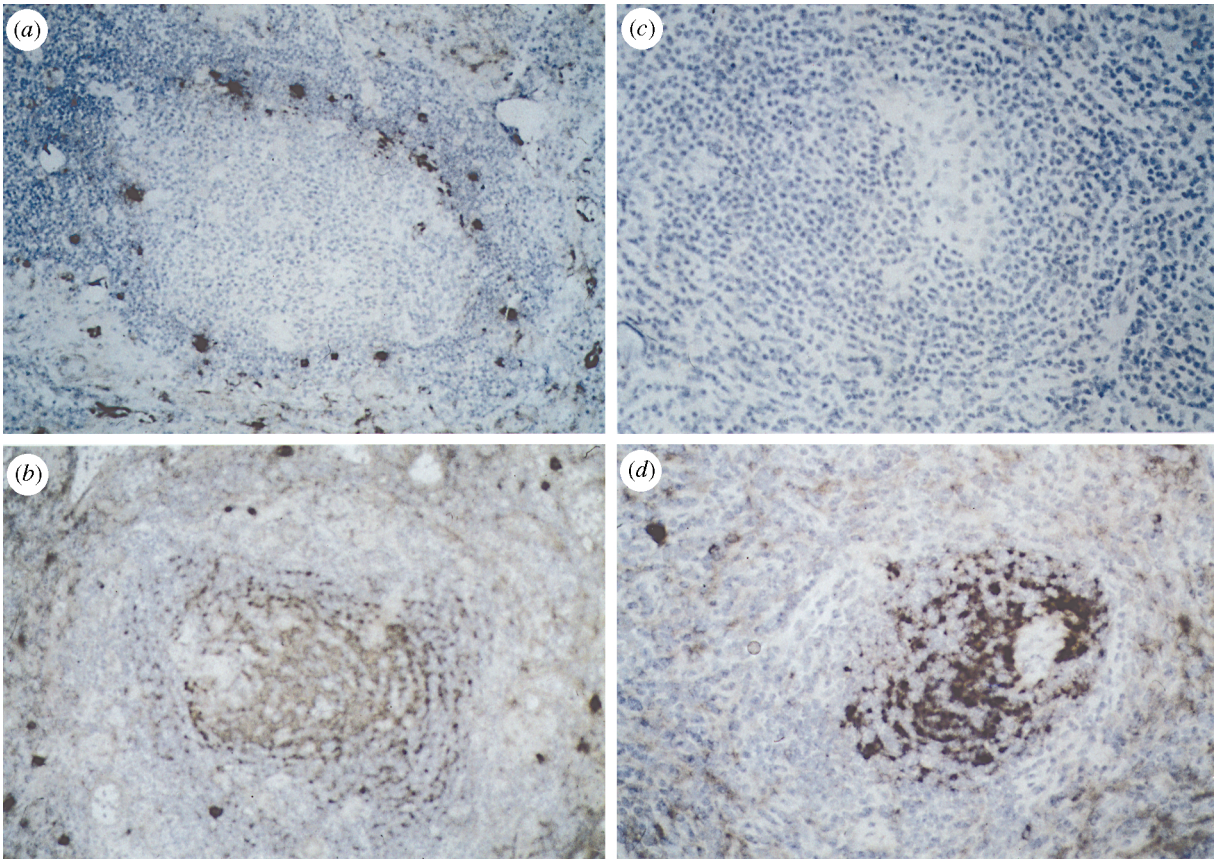

Figure 6. Photomicrograph of CD lymph nodes after immunostaining for vIL-6 (*a*,*c*) and hIL-6 (*b*,*d*) proteins. The germinal centres (GCs) in (*a*,*b*) are infected with KSHV while the GCs in (*c*,*d*) are from KSHV-negative CD patients. Viral IL-6 is abundantly expressed (*a*) in the KSHV-infected GC, with the majority of infected cells lying in the marginal zones, whereas no vIL-6 staining is present in the KSHV-free GC (*c*). In contrast, hIL-6 expression is diminished and mainly localized to the central regions of the KSHV-infected GC and scattered marginal zone cells (*b*) but is abundantly expressed in the central region of the KSHV-negative specimen (*d*). For both KSHV-infected and KSHV-free CD, vIL-6 or hIL-6 is expressed only by a minority of cells, while the bulk of the tumour is formed from paracrine effects of these cytokines on B-cell proliferation. Reproduced with permission from Parravicini (1997*a*).

transcription. Viral IRF1 normally sequesters transcription coadaptors away from promoters, thereby shutting off transcription. Viral IRF1 binding to CBP, however, appears to recruit this HAT coadaptor to the *c-myc* promoter, resulting in activation. Presumably, a cellular factor directly binding to the *c-myc* promoter specifically recruits the vIRF1–CBP complex. Upregulation of *c-myc* is required for cell transformation (Jayachandra *et al.* 1999), and interference with these IFN-regulated pathways explains in part why this viral protein transforms rodent cells. Although EBNA-2 and E1A have no sequence similarity to vIRF1, they also activate the *c-myc* promoter using a similar mechanism (Jayachandra *et al.* 1999).

(e) *MHC antigen regulation*

In contrast to the innate immune system, adaptive immunity requires processing of viral peptides through MHC-regulated pathways in order to generate a specific anti-KSHV immune response. Antigen presentation through the MHC class I pathway requires generation of proteolytically cleaved viral peptides, which are loaded onto the MHC heavy and light chain dimer in the endoplasmic reticulum (ER). Peptide loading onto the nascent MHC class I dimer stabilizes it, and it is transported to the outer membrane from the ER through the secretory pathway (reviewed in Ploegh 1998). In this way, infected cells continually process and present viral peptides to antigen-specific receptors on $CD8^+$ CTLs. Several viruses have evolved mechanisms to inhibit antigen presentation by MHC molecules. To abort this response, herpes simplex virus 1 and 2 ICP47 and cytomegalovirus (CMV) US6 proteins inhibit the ER antigen transporter responsible for peptide loading onto MHC (Hill *et al.* 1995; Ahn *et al.* 1997). Adenovirus E3 and CMV US3 proteins act after peptide loading by retaining the MHC–peptide complex in the ER and preventing antigen presentation to the extracellular milieu.

A novel viral CTL escape mechanism for KSHV was discovered by Coscoy & Ganem (2000), who cloned the KSHV K genes into retroviral vectors to systematically search for MHC downregulators. By screening HeLa cells expressing the KSHV proteins for MHC class I antigen expression using flow cytometry, this group found that two KSHV genes, *ORF K3* and *ORF K5*, encoded proteins that inhibited surface expression of MHC class I. Pulse-chase analysis reveals that these proteins accelerate enhanced endocytosis and retention of surface MHC molecules. These results have been confirmed and extended by several groups (Ishido *et al.* 2000; Lee *et al.* 2000). In line with these observations, CTL killing of PEL cells is

markedly diminished compared with that of EBV-infected Burkitt's lymphoma cell lines (Brander *et al.* 2000).

The immune system, however, has apparently evolved a counter-response to deal with viral interference with MHC antigen presentation. Loss of surface MHC expression on a cell (in combination with the presence of other specific cell-membrane proteins) activates non-specific cytotoxic natural killer (NK) cells. An attractive possibility is that this is in part prevented by KSHV vFLIP, which inhibits the Fas-induced apoptosis by surveillance NK cells. Whether or not this complementary relationship exists for K3, K5 and vFLIP proteins *in vivo* remains to be investigated.

(f) *KSHV cytokines, chemokines and chemokine receptor*

KS research has frequently focused on the role of cytokines in driving spindle-cell proliferation. Early studies performed before the discovery of KSHV often used KS lesion-derived cell lines, which did not harbour KSHV and are of unclear relevance to the tumour. However, *in situ* studies demonstrate marked elaboration of inflammatory cytokines directly in tumour tissues (Fiorelli *et al.* 1998), and considerable interest exists in both the virus-encoded cytokines and cellular cytokines induced by KSHV infection.

The best-studied KSHV cytokine is the vIL-6 protein encoded by *ORF K2* (Moore *et al.* 1996*a*; Neipel *et al.* 1997*a*; Nicholas *et al.* 1997). Viral IL-6 is synthesized into secretory granules, like its cellular counterpart, and secreted from KSHV-infected haematopoietic cells. It is not appreciably expressed in most KS lesions (Moore *et al.* 1996*a*; Parravicini *et al.* 1997*a*; however, see Cannon *et al.* 1999*b*), but immunohistochemistry can readily detect elaboration of vIL-6 in both CD and PEL tumours (Parravicini *et al.* 1997*a*, 2000).

Viral IL-6 has 25% sequence identity to human IL-6 (hIL-6), particularly in regions possessing contact points for the gpl30 receptor, which is responsible for signal transduction for members of the IL-6 cytokine family. In the case of hIL-6, interaction with the gp80 (or IL-6 receptor-α) coreceptor is required for hIL-6 to activate gpl30 signalling. Viral IL-6, however, is able to directly activate gpl30 signalling without coreceptor usage (Molden *et al.* 1997), suggesting that it may have a broader set of target effector cells than the human cytokine. This has recently been confirmed using a variety of cell types (Hoischen *et al.* 2000; Mullberg *et al.* 2000). Mutation analyses also demonstrate that replacement of conserved residues involved in gp80 interaction for the human cytokine have little effect on the activity of vIL-6 (Wan *et al.* 1999). It is likely, however, that gp80 interactions with vIL-6 increase the efficiency of signalling without being absolutely required.

Despite these differences in receptor usage, no clear differences have yet been found in downstream signalling pathways between hIL-6 and vIL-6 (Osborne *et al.* 1999). Like hIL-6, vIL-6 activates STAT-1, STAT-3 and STAT-5 transcription factors (Molden *et al.* 1997; Burger *et al.* 1998; Wan *et al.* 1999) and activates a Ras-mitogen-activated protein kinase pathway through receptor activation (Osborne *et al.* 1999; Wan *et al.* 1999). These findings suggest that either vIL-6 has a broader target cell profile than hIL-6, or that vIL-6 can escape downregulation of gp80 as a means of inhibiting IL-6-like signalling activity. Downstream effects of these signalling cascades are likely to include induction of anti-apoptotic proteins and inhibition of cell-cycle checkpoint proteins (Schwarze & Hawley 1995; Urashima *et al.* 1997).

Functionally, both hIL-6 and vIL-6 induce B-cell proliferation and prevent apoptosis in susceptible cell lines (Moore *et al.* 1996*a*; Nicholas *et al.* 1997; Burger *et al.* 1998). Although contradictory results exist (Asou *et al.* 1998), it appears that PEL cells in tissue culture are autocrine-dependent on vIL-6, hIL-l0 and nerve growth factor, but not hIL-6, for growth and proliferation (Jones *et al.* 1999; Pica *et al.* 2000). Unlike other latency expressed genes (such as LANA-1 and LANA-2), vIL-6 secretion occurs only in a minority of cells, which could be due to local levels of cellular cytokine (e.g. IFN) activation. This raises the intriguing possibility that the vIL-6 autocrine circuit present in PEL cells represents a viral response to antiviral defences activated within the host cell. Human IL-6 plays a significant role in maintenance of EBV-infected lymphoblastoid cell lines and is induced both by gp350/220 binding to CR2 and by LMP-1 signalling.

Although vIL-6 is only expressed in a portion of PELs and CD tumour cells, it may have widespread effects since it is a secreted cytokine and is likely to play an important role in the pathogenesis of these diseases. In the case of CD, only about half of these tumours are infected with KSHV, with the remaining tumours being due to excess secretion of hIL-6 in the absence of KSHV infection. Among those CD tumours infected with KSHV, however, vIL-6 appears to be responsible for proliferation of the uninfected B cells that make up the bulk of the tumour (figure 6). Viral IL-6 also activates secretion of vascular endothelial growth factor (VEGF) (Aoki *et al.* 1999) and enforced overexpression of vIL-6 in NIH3T3 cells results in cell transformation and malignant tumour formation.

KSHV has been implicated as a cause of multiple myeloma. This cancer is clearly paracrine-dependent on cellular IL-6, and some evidence has been generated to suggest that KSHV infection of stromal cells in the tumour and elaboration of vIL-6 may play a role in its pathogenesis (Rettig *et al.* 1997). Extensive follow-up studies have failed to find any connection between KSHV and multiple myeloma tumours using a variety of assay techniques (see Whitby *et al.* 1997; Parravicini *et al.* 1997*b*; Olsen *et al.* 1998; Tarte *et al.* 1998). There is no increase in myeloma incidence where KSHV infection is highly prevalent (Whitby *et al.* 1997). There is a weak epidemiological association between KS and myeloma in US cancer registry data, but most of these KS tumours occur secondarily to primary onset of myeloma, a pattern consistent with the use of immunosuppressive drugs (e.g. dexamethasone) in myeloma treatment that might predispose to KS among KSHV-infected persons (Cannon *et al.* 2000). Nonetheless, a real possibility remains that KSHV vIL-6 contributes to a minority of multiple myeloma and Waldenström macroglobulinaemia cases and that this relationship is obscured by the large bulk of cases in which KSHV does not play any role.

In addition to vIL-6, KSHV encodes three secreted chemokines (chemoattractant cytokines) that have unique properties. These small 10 kDa proteins, macrophage inflammatory protein (MIP)I (vMIP-1a), vMIP-II (vMIP-1b) and vMIP-III (BCK), are encoded by *ORF K4*, *ORF 4.1* and *ORF 6*, respectively, located at the left end of the LUR (Moore *et al.* 1996*a*; Nicholas *et al.* 1997; Russo *et al.* 1996; Neipel *et al.* 1997*b*). Chemokines are grouped according to the presence of a dicycsteine motif (either CC or CXC; other single-cysteine chemokines also exist), which binds to corresponding GPCRs. The three KSHV chemokines are agonists at specific receptors and belong to the CC chemokine family. Viral MIP-I is a CC receptor 8 (CCR8) agonist (Dairaghi *et al.* 1999; Endres *et al.* 1999), vMIP-II binds and activates CCR3 (Boshoff *et al.* 1997) and vMIP-III is an agonist for CCR4 (Stine *et al.* 2000). Unlike most cellular chemokines, KSHV chemokines may also bind a variety of receptors (including CXC receptors), acting as antagonists (Kledal *et al.* 1997).

Because of their ability to induce chemotaxis in specific cell types, early speculation on the function of these viral proteins leaned towards the possibility that they serve to attract host cells to enhance virus transmission. This is unlikely to be the case since the estimated KSHV infection rate for most cell populations (e.g. circulating B lymphocytes) is $1{:}10^3$ to $1{:}10^6$. It is difficult to see how the chemokines could significantly increase the proportion of target cells for infection under these circumstances. A more likely function is found by examining receptors activated by the KSHV chemokines. All three major receptors, CCR3, CCR4 and CCR8, are chemoattractant receptors for Th2 lymphocytes. Inhibition of Th1 immune responses might be achieved by activating these receptors and recruiting Th2 cells to sites of infection (Sozzani *et al.* 1998; Endres *et al.* 1999; Stine *et al.* 2000). This is a novel mechanism for polarization of Th2 immune responses, a common immunological defence mechanism used by a variety of viruses. One experiment demonstrating the capacity of these immunomodulatory viral proteins by DeBruyne *et al.* (2000) demonstrated that virus-mediated gene transfer of vMIP-II into cardiac allografts enhanced donor tissue survival by inhibiting a specific cell-mediated immune response. Surprisingly, short peptides derived from vMIP-I and vMIP-II may be biologically active in some *in vitro* assays and are attractive candidates as small molecule therapeutics (Benelli *et al.* 2000).

In addition to inhibiting Th1 immunity, all three viral chemokines induce a strong angiogenic response (Boshoff *et al.* 1997; Stine *et al.* 2000). This is unlikely to contribute to KS pathogenesis, with the possible exception of vMIP-III, which is the only chemokine significantly expressed in KS lesions, but all three chemokines might potentially contribute to the vascular component present in CD tumours. The potential for the chemokines to inhibit HIV entry has also been explored (Moore *et al.* 1996*a*; Boshoff *et al.* 1997; Stine *et al.* 2000; Hibbitts *et al.* 1999).

KSHV possesses a chemokine receptor in addition to the three secreted chemokines. KSHV *ORF 74* encodes vGPCR that belongs to the CXC chemokine receptor family (Cesarman *et al.* 1996). Viral GPCR is a seven-transmembrane-spanning protein with greatest similarity to the IL-8 receptor. When vGPCR is stably expressed in NIH3T3 cells, it induces full cell transformation and secretion of VEGF (Arvanitakis *et al.* 1997; Bais *et al.* 1998). Like vIL-6, vGPCR is not appreciably expressed in most KS spindle cells, although it is present in the small minority of cells undergoing lytic replication (Kirshner *et al.* 1999). Transgenic mice expressing vGPCR under control of the haemopoietic cell CD2 promoter develop diffuse endothelial cell tumours resembling KS. These tumours appear to be at least in part derived from a paracrine effect due to haemopoietic cell expression of the vGPCR (Cesarman *et al.* 2000). This has led to the suggestion that the minority of cells actively undergoing lytic replication and expressing vGPCR might induce the KS phenotype in surrounding latently infected spindle cells.

Signalling studies demonstrate that the vGPCR is constitutively active under standard tissue culture conditions (Arvanitakis *et al.* 1997; Ho *et al.* 1999) but can be further activated by IL-8 and GRO-α (Gershengorn *et al.* 1998; Rosenkilde *et al.* 1999). In contrast, the IFN-induced chemokine IP-10, vMIP-II and SDF-1 inhibit vGPCR signalling (Geras-Raaka *et al.* 1998*a*,*b*). Viral GPCR has an alteration in a conserved DRY motif responsible for signal regulation in GPCRs (Burger *et al.* 1999). When the DRY motif of the cellular homologue of vGPCR (CXCR2) is mutated to the vGPCR sequence (VRY), the cellular protein is also constitutively active and able to induce cell transformation. Activation of the receptor results in a Ca^{2+} influx and stress-activated protein kinase pathway activation through the phosphoinositide-inositol 1,4,5-trisphosphate pathway (Arvanitakis *et al.* 1997).

(g) *KSHV proteins involved in BCR pathway activation*

Both the far left and right hand ends of the LUR show considerable sequence variability between KSHV isolates. The first recognizable ORF, *ORF K1*, encodes a 46 kDa transmembrane glycoprotein possessing a cytoplasmic immunoreceptor tyrosine activation motif (ITAM) involved in BCR and T-cell-receptor signalling. This ITAM-like domain has been shown to transduce cellular activation signals via phosphorylation of its tyrosine residues (Lee *et al.* 1998*a*). Upon tyrosine phosphorylation, classical BCR signalling pathways, including syk and phospholipase C γ_2 phosphorylation, have been shown to be activated to initiate calcium-dependent signal transduction in B cells (Lagunoff *et al.* 1999). Unlike cellular ITAM-containing proteins, which require exogenous ligand cross-linking for activation, the ectodomain of *ORF K1*, possibly by homomultimerization, has constitutive signalling properties (Lagunoff *et al.* 1999). In addition to presenting a constitutively active BCR-like protein on the surface of infected cells, the KSHV ORF K1 protein also appears to block the intracellular transport of BCR complexes to the cell surface (Lee *et al.* 2000). K1 protein has been shown to cause transformation when expressed in rodent fibroblasts, and can functionally replace the HVS saimiri-transforming protein in induction of T-cell lymphomas by a chimeric virus in common marmosets (Lee *et al.* 1998*b*).

The genomic region between *ORF 75* and the TR is highly divergent between two prototypical isolates of

KSHV and has been designated as *P* (for predominant) and *M* (for minor). Despite this sequence variation, a similar organization of eight exons defines a family of alternatively spliced transcripts, which have a class II pattern of expression (Glenn *et al.* 1999). Depending on variable donor–acceptor splice-site usage, the predicted protein products, designated latency-associated membrane proteins (LAMPs), may consist of up to 12 transmembrane domains, and a hydrophilic C-terminal cytoplasmic tail. The large *P* and *M* versions of the predicted proteins have an overall amino-acid identity of only 29%, with 46% similarity (Glenn *et al.* 1999). Although numerous combinations of exons have been detected, the hydrophilic tail, encoded by exon 8, is retained in all splice variants. This cytoplasmic region of KSHV LAMP contains both a group III tyrosine motif as well as an SRC phosphotyrosine kinase SH2-binding motif also present in the cytoplasmic domain of EBV LMP-2A. Additionally, it contains a PFQPADE motif similar to the tumour necrosis factor receptor-associated factor (TRAF)-binding motif within the C-terminal activating region 1 (CTAR-1) of LMP-1. GST pull-down assays demonstrate that the C-terminal cytoplasmic domain of KSHV LAMP can bind TRAF-1, TRAF-2, and TRAF-3 (Glenn *et al.* 1999). Expression of genomic as well as cDNA-tagged LAMP constructs show patchy cytoplasmic membrane localization of LAMP reminiscent of that seen with EBV LMP-1 (Glenn *et al.* 1999). In contrast to K1, LAMP overexpression blocks calcium influx in response to BCR activation from cross-linking antibodies (Choi *et al.* 2000). This response is similar to EBV LMP-2A. Taken together, the K1 and LAMP proteins share many of the properties of EBV LMP-1 and LMP-2A.

4. FUNCTIONAL INTERPLAY OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS REGULATORY PROTEINS IN IMMUNITY AND TUMORIGENESIS

The KSHV genomic sequence provides an exciting and interesting set of clues to long-standing questions on virus–host cell interactions. One immediate conclusion that can be drawn is that KSHV recapitulates many of the functions of other viruses, particularly EBV. This contributes to the general sense that there are fundamental properties shared among phylogenetically divergent viruses. Not only has this led to the discovery that KSHV targets cellular proteins targeted by other viruses (Friborg *et al.* 1999), but it also allows for predictive experiments to find novel functions among unrelated viruses for properties first discovered using KSHV genes (Jayachandra *et al.* 1999). Determining the extent of functional similarities, as well as dissimilarities, between KSHV and other viruses has led to major insights into cell-type-specific interactions, viral life cycles and viral disease pathogenesis.

Many of the KSHV-regulatory genes can be interpreted to play a functional role in controlling cellular antiviral innate and adaptive immune responses. Viral IRF, vFLIP, vIL-6 as well as K3 and K5 proteins directly affect established immune-response pathways. The immunoreceptor-regulatory proteins, ORF K1, LAMP and ORF 4, are also likely to have a direct impact on immune responses that are particular to B cells. These immune pathways not only affect viral replication, but also regulate cell-cycle control, apoptosis and tumour cell immune surveillance. It is understandable then that the primary functions of some KSHV 'oncoproteins' such as ORF K1, vIL-6 and vIRF, are to alter host-cell immune responses, but these proteins cause cell transformation *in vitro* as well. This raises the possibility that cellular proteins and pathways that are not normally thought of as having an immune function, such as pRB1 and p53, actually are critical in preventing successful persistent viral infections (Moore & Chang 1998).

It still is too early to say with certainty which genes or combination of genes contribute to KSHV-induced human tumours. Likely candidates include LANA-1, vCYC and vFLIP for KS lesions as well as LANA-2 for PEL due to their persistent expression patterns in these tumours. There is the unique possibility that paracrine effects, particularly through the vGPCR, contribute to tumour cell growth as well. For hyperplastic lymphoproliferative disorders such as multicentric CD, a variety of viral genes is likely to contribute to lymphocyte proliferation, although vIL-6 is apparently the primary viral protein responsible for this pathology. Studies to examine these and other questions in KSHV research are certain to shed light on pathological processes occurring in other viral infections as well.

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