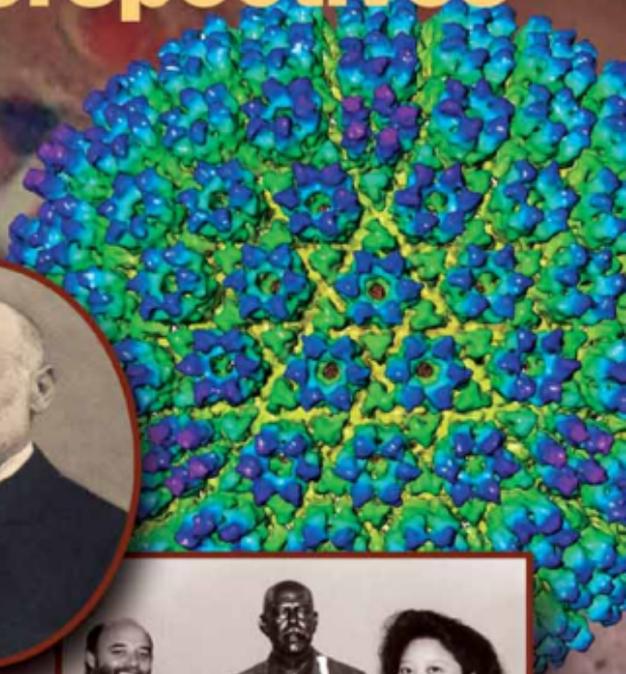


C. Boshoff R.A. Weiss (Eds.)

Kaposi Sarcoma Herpesvirus: New Perspectives



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C. Boshoff and R. A. Weiss (Eds.)

Kaposi Sarcoma Herpesvirus: New Perspectives

With 29 Figures and 14 Tables

 Springer

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Cover Illustration:

Juan Chang and Patrick Moore receiving the Koch Prize for their discovery of Kaposi's sarcoma-associated herpesvirus in 1994. Also shown is a KSHV virion from chapter by O'Connor and Kedes, p. 43. and a photograph of Moritz Kaposi. Background shows an histological image of a Kaposi's sarcoma tumor.

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Preface

Moritz Kaposi (born Moritz Kohn 1837, Kaposvár, Hungary; died 1902, Vienna) succeeded his father-in-law, Ferdinand von Hibra (1816–1880) to become one of the foremost dermatologists in the German-speaking world. His remarkable clinical acumen is evident from his descriptions of systemic lupus erythematosus (1869), idiopathic multiple pigmented sarcoma of the skin (1872 Kaposi sarcoma), and xeroderma pigmentosum (1882). His name change from Kohn to Kaposi (1871) reflects his professional ambitions to be distinguished from the other *Kohns* working in the Vienna Medical Faculty at the time. Although we now associate Kaposi sarcoma occurring in eastern European and Mediterranean populations (*classic Kaposi*) with an indolent disease, the cases first described by Kaposi had an aggressive clinical course, including some with fatal systemic disease. Underlying illness (*krankheit*) might have contributed to a more immunosuppressive state (Kaposi 1872).

From the 1940s onwards, various workers observed similar vascular tumours in sub-Saharan Africa, including an aggressive lymphadenopathy in children (endemic Kaposi) (Ackerman 1962; Oettle 1962). It remains to be established whether this lymphadenopathic disease in African children represents primary infection with Kaposi sarcoma-associated herpesvirus (KSHV). In the 1970s, the introduction of organ transplantation with its related iatrogenic immunosuppression resurrected this tumour as an important cause of morbidity and even mortality (Penn 1979). The Italian dermatologist Giraldo suggested in 1972 that herpesviruses could be involved in the pathogenesis of Kaposi sarcoma, after they showed by electron microscopy herpes-type viral particles in cells cultured from these tumours (Giraldo 1972). They subsequently identified these virions as cytomegalovirus.

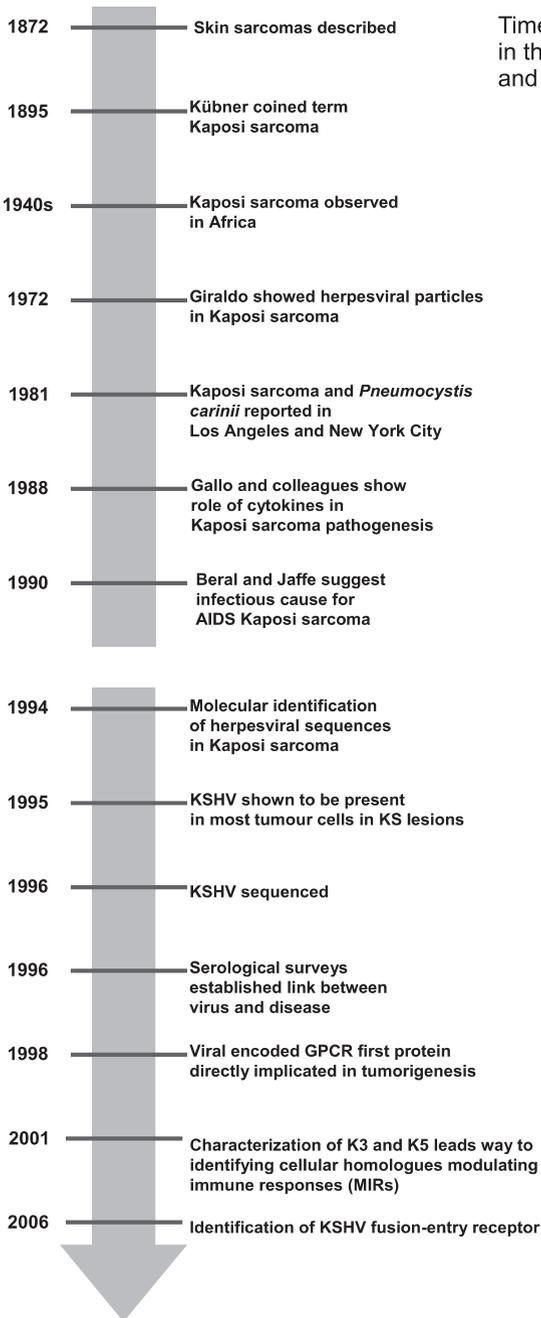
The reports from New York City and Los Angeles in 1981 of Kaposi sarcoma and *Pneumocystis carinii* pneumonia by the Centers for Disease Control in Atlanta heralded the AIDS pandemic (Friedman-Kien 1981). In 1981, about 48% of gay men with AIDS presented with or developed Kaposi sarcoma during the course of their illness. The incidence of AIDS-related Kaposi sarcoma in 1990 in this population was less than 15%. It is still unclear whether this is mainly

due to a fall in the seroprevalence of Kaposi sarcoma-associated herpesvirus (KSHV or HHV8) in this population, or whether these were related to other disease-associated risk factors. The particular symmetrical distribution of AIDS-related Kaposi sarcoma on the skin, high rate of mucosal membrane, lymph node and visceral involvement, provoked various hypotheses regarding the pathogenesis of this disease: tissue-specific temperature or oxygen tensions might favor KS tumour cell proliferation, or attraction of infected cells by specific chemokine-rich environments could favor these sites of disease. The demonstration that solid epithelial cancer micrometastases could be attracted to specific organs by chemokines corresponding to the specific chemokine receptors expressed on such cancer cells, favors a hypothesis where circulating Kaposi tumour cells, or KSHV-infected cells, home-in on specific organs. Underlying HIV infection might influence the distribution of such chemokines and certainly promotes the aggressiveness of the disease.

Robert Gallo and his team were among the first to culture cells from AIDS-related Kaposi sarcoma, and they introduced the concept of an autocrine and paracrine driven tumour, where cytokines stimulate the growth of this malignancy (Salahuddin 1988; Ensoli 1989). A contemporary of Kaposi, Virchow proposed in 1863 that cancerous tumours occur at sites of chronic inflammation (Balkwill 2001) and in 1986 Dvorak speculated that tumours are wounds that do not heal (Dvorak 1986). The model of a tumour landscape, where inflammatory cells produce cytokines and other growth factors which contribute to the initiation and maintenance of tumour growth has since become a major focus of cancer research. Interestingly, Kaposi sarcoma lesions also display the Kübner phenomenon (tumour develops at sites of an old injury).

Epidemiological studies by Harold Jaffe and Valerie Beral in the early years of the AIDS epidemic suggested that an infectious agent other than HIV was the culprit causing Kaposi sarcoma (Beral 1990). However, their suggestion that such an infection is mainly transmitted by the faecal-oral route was probably incorrect.

Patrick Moore (epidemiologist to the City of New York), and his wife Yuan Chang (pathologist at Columbia University), used representational difference analysis to identify sequences of a new herpesvirus in 1994 (Chang 1994). The technique of representational difference analysis was first described by Lisitsyn and Wigler (Lisitsyn 1993). They employed this polymerase chain reaction subtraction technique to successfully identify a sequence that clearly belonged to a herpesvirus genome, but was distinct from previously characterized herpesviruses. KSHV is the second human oncogenic virus to be identified by molecular techniques, the first being human cervical papillomavirus in 1983 by Harald zur Hausen and colleagues.



Time Line: Important events
in the history of Kaposi sarcoma
and its associated virus

Since the identification of two sequences from KSHV, we have learned much about this pathogen as reflected in this book. As it turned out, KSHV itself encodes for a number of cytokines, and induces cellular cytokine secretion, contributing to tumour growth. Moreover, KSHV vFLIP targets the IKK-NF κ B axis to encourage the inflammatory microenvironment observed by Gallo and his colleagues. KSHV continues to elucidate both mechanisms of viral oncogenesis, and cellular and immune pathways involved in non-viral driven neoplasia.

We bring together here various experts in their respective fields, addressing some key aspects of KSHV biology, and we are grateful for their contributions.

University College London

Chris Boshoff and Robin A. Weiss

Table 1 Twenty of the most cited articles on Kaposi sarcoma herpesvirus

Article	Estimated number of citations (2006)
1. Chang Y et al. (1994) Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. <i>Science</i> 266:1865–1869	1950
2. Cesarman E et al. (1995) Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. <i>NEJM</i> 332:1186–1191	880
3. Russo JJ et al. (1996) Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). <i>PNAS</i> 93:14862–14867	735
4. Whitby D et al. (1995) Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. <i>Lancet</i> 346:799–802	570
5. Moore PS et al. (1995) Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and without HIV infection. <i>NEJM</i> 332:1181–1185	555
6. Gao SJ et al. (1996) KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. <i>Nat Med</i> 2:925–928	527
7. Moore PS et al. (1996) Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. <i>Science</i> 274:1739–1744	505
8. Renne R et al. (1996) Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. <i>Nat Med</i> 2:342–346	499

Table 1 (continued)

Article	Estimated number of citations (2006)
9. Kedes DH et al. (1996) The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): Distribution of infection in KS risk groups and evidence for sexual transmission. <i>Nat Med</i> 2:918–924	464
10. Boshoff C et al. (1995) Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. <i>Nat Med</i> 1:1274–1278	415
11. Simpson GR et al. (1996) Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen. <i>Lancet</i> 348:1133–1138	403
12. Bais C et al. (1998) G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. <i>Nature</i> 391:86–89	390
13. Lennette ET et al. (1996) Antibodies to human herpesvirus type 8 in the general population and in Kaposi's sarcoma patients. <i>Lancet</i> 348:858–861	365
14. Gao SJ et al. (1996) Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. <i>NEJM</i> 335:233–241	351
15. Moore PS et al. (1996) Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. <i>J Virol</i> 70:549–558	351
16. Rettig MB et al. (1997) Kaposi's sarcoma-associated herpesvirus infection of bone marrow dendritic cells from multiple myeloma patients. <i>Science</i> 276:1851–1854	346
17. Nador RG et al. (1996) Primary effusion lymphoma: A distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpesvirus. <i>Blood</i> 88:645–656	346
18. Staskus KA et al. (1997) Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (Spindle) tumor cells. <i>J Virol</i> 71:715–719	292
19. Boshoff C et al. (1997) Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. <i>Science</i> 278:290–294	279
20. Kledal TN et al. (1997) A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. <i>Science</i> 277:1656–1659	249

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12. Friedman-Kien AE, Laubenstein L, Marmor M et al. (1981) Kaposi's sarcoma and pneumocystis pneumonia among homosexual men – New York and California. *Morbidity Mortality Rep* 30:305–308
13. Gao SJ, Kingsley L, Hoover DR, Spira TJ, Rinaldo CR, Saah A, Phair J, Detels R, Parry P, Chang Y, Moore PS (1996) Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. *New Engl J Med* 335:233–241
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16. Kaposi M (1872) Idiopathiches multiples pigment sarcom der Haut. *Archiv fur Dermatologie Syphilis* 4:265–272
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List of Contents

Modern Evolutionary History of the Human KSHV Genome	1
<i>G. S. Hayward and J.-C. Zong</i>	
Rhesus Monkey Rhadinovirus: A Model for the Study of KSHV	43
<i>C. M. O'Connor and D. H. Kedes</i>	
The Rta/Orf50 Transactivator Proteins of the Gamma-Herpesviridae	71
<i>M. R. Staudt and D. P. Dittmer</i>	
Structure and Function of Latency-Associated Nuclear Antigen	101
<i>S. C. Verma, K. Lan, and E. Robertson</i>	
The KSHV and Other Human Herpesviral G Protein-Coupled Receptors	137
<i>M. Cannon</i>	
Regulation of KSHV Lytic Gene Expression	157
<i>H. Deng, Y. Liang, and R. Sun</i>	
Kaposi Sarcoma Herpesvirus-Encoded Interferon Regulator Factors	185
<i>M. K. Offermann</i>	
Endothelial Cell- and Lymphocyte-Based In Vitro Systems for Understanding KSHV Biology	211
<i>S. C. McAllister and A. V. Moses</i>	
KSHV After an Organ Transplant: Should We Screen?	245
<i>A.-G. Marcelin, V. Calvez, and E. Dussaix</i>	
Kaposi Sarcoma-Associated Herpesvirus and Other Viruses in Human Lymphomagenesis	263
<i>E. Cesarman and E. A. Mesri</i>	
The Use of Antiviral Drugs in the Prevention and Treatment of Kaposi Sarcoma, Multicentric Castleman Disease and Primary Effusion Lymphoma	289
<i>C. Casper and A. Wald</i>	

Interactions Between HIV-1 Tat and KSHV 309
Y. Aoki and G. Tosato

Subject Index 327

List of Contributors

(Addresses stated at the beginning of respective chapters)

Aoki, Y. 309

Calvez, V. 245

Cannon, M. 137

Casper, C. 289

Cesarman, E. 263

Deng, H. 157

Dittmer, D. P. 71

Dussaix, E. 245

Hayward, G. S. 1

Kedes, D. H. 43

Lan, K. 101

Liang, Y. 157

Marcelin, A.-G. 245

McAllister, S. C. 211

Mesri, E. A. 263

Moses, A. V. 211

O'Connor, C. M. 43

Offermann, M. K. 185

Robertson, E. 101

Staudt, M. R. 71

Sun, R. 157

Tosato, G. 309

Verma, S. C. 101

Wald, A. 289

Zong, J.-C. 1

Modern Evolutionary History of the Human KSHV Genome

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1	Introduction	2
2	KSHV Genetic Variability	4
2.1	Early Studies of VIP Hypervariability Patterns	4
2.2	TMP Allelic Variability and Chimeric Genes	5
2.3	More Recent Comprehensive Analyses Including the Central Constant Segment	6
3	Phylogenetics of the Hypervariable VIP or K1 Genes	7
3.1	Age and Origin of B-Subtype VIP Genes in Sub-Saharan Africa	10
3.2	The African A5 Cluster of VIP Genes	12
3.3	Source and Origin of the Novel Ugandan VIP-F and VIP-C7 Genes	13
3.4	A Novel E-Subtype VIP Gene from a Taiwan Aborigine	14
4	Variable Sequence Loci within the Constant Region	15
4.1	Clustering, Subtyping, and Linkage	16
4.2	Subtype Distribution Patterns Within the Constant Region	16
4.3	The 12 Major Genotype Patterns in KSHV	17
4.4	Geographic Distribution of Constant Segment Subtypes	19
5	The TMP-P and TMP-M Alleles and Adjacent Chimeric Regions	22
5.1	Relative Lack of Variation Within UPS75 and the TMP Coding Regions of the Eurasian Subtypes of the TMP-P and TMP-M Alleles	22
5.2	Distinctive African Versions of TMP-P	24
5.3	Distinctive African Versions of TMP-M	24
5.4	Distinctive Pacific Rim Versions of Both the TMP-P and TMP-M Alleles	25
5.5	Evidence for Three Distinct Chimeric Boundaries in African KSHV Genomes with TMP-M Alleles	26
6	The Rare KSHV N Alleles in Southern Africa	28
6.1	Additional N-Subtype Genomes at the UPS75 Locus	28
6.2	Novel N-Allele KSHV TMP Genes from Southern Africa	28
6.3	Extension into the ORF75E Locus Reveals Additional RHS Chimeric Junctions	30

7	Differential Evolutionary History of Segments Along the KSHV Genome	31
7.1	Age of Divergence and Origin of the Three TMP Alleles	31
7.2	Timing of the P/M Chimeric Events	33
7.3	Source of the Exotic TMP-N Alleles	34
7.4	Only Modern Versions of the VIP Genes Have Been Detected	35
7.5	Source and Rapid Penetrance of the Anomalous A5 VIP Subtype Within Sub-Saharan Africa	36
8	Overall Significance	37
8.1	Precedents From Other Herpesviruses	37
8.2	Contribution of Chimeric Fixation to Herpesvirus Genomic Evolution . . .	38
	References	39

Abstract The genomes of several human herpesviruses, including Kaposi sarcoma (KS) herpesvirus (KSHV), display surprisingly high levels of both genetic diversity and clustered subtyping at certain loci. We have been interested in understanding this phenomenon with the hope that it might be a useful diagnostic tool for viral epidemiology, and that it might provide some insights about how these large viral genomes evolve over a relatively short timescale. To do so, we have carried out extensive PCR DNA sequence analysis across the genomes of 200 distinct KSHV samples collected from KS patients around the world. Here we review and summarize current understanding of the origins of KSHV variability, the spread of KSHV and its human hosts out of Africa, the existence of chimeric genomes, and the concept that different segments of the genome have had different evolutionary histories.

1

Introduction

Kaposi sarcoma herpesvirus (KSHV) (or HHV8) is a gamma-2 or rhadinovirus subfamily herpesvirus (Chang et al. 1994; Russo et al. 1996) that is believed to be the etiological agent of Kaposi sarcoma (KS), primary effusion lymphoma (PEL), and some forms of multicentric Castleman disease in humans. Importantly, both classic KS and long-term inapparent latent infection by KSHV are relatively rare in extant human populations within the USA, Europe, and Asia (approx 1% seropositivity) but are significantly more common in the Mediterranean area (5%–15%) and most common in sub-Saharan Africa (50% or more). Presumably because of reduced immunosurveillance, the incidence of KS increases 500-fold in iatrogenic organ transplant patients and up to 20,000 fold in homosexual AIDS patients in the USA and Europe, and KS is now the most common human tumor found in southern Africa (Chokunonga et al. 1999; Dedicoat and Newton 2003).

Our study of genotypic variation among KSHV genomes derived from different human population groups on different continents has proceeded in three stages. First, we generated a simplistic “ethnic migration” model based only on sequencing of the hypervariable ORF-K1 or VIP genes at the LHS end of the genome (Kajumbula et al. 2006; Zong et al. 1999; Zong et al. 2002). Second, we reported the discovery and analysis of additional complexity and chimerism at the extreme RHS of the genome, associated with the concept of multiple alleles of the ORF-K15 or TMP gene (Poole et al. 1999; Zong et al. 2002) (Zong, J.C., Su, I.J., Morris, L., Alagiozoulou, L., Sitas, F., Kajumbula, H., Katange-Mbide, E., Boto, W and Hayward, G.S., manuscript in preparation). Third, we have also now carried out an exhaustive analysis of multiple loci across the central constant segment of the genome (Zong, J-C and Hayward, G.S., unpublished data). Each stage and segment gave rather different results but a reasonably complete picture of the overall pattern of variability within and across the whole length of modern human KSHV genomes has now emerged. These data permit deductions about the history and origin of different parts of the KSHV genome and provide for the first time some important insights into how a large DNA virus genome evolves within the relatively short evolutionary time frame of the divergence of both premodern humanoid species and of modern *Homo sapiens*. In particular, we will introduce the concept of both “old” and “new” segments of the genome and of the role of rare interspecies chimerism, as well as how founder and bottleneck effects associated with modern human migration patterns have impacted the genome.

A critical aspect of this story for the KSHV genome is the realization that the situation observed here is dramatically different from that found in most other more typical herpesviruses (e.g., HCMV). In particular, multiple infections and intragenomic recombination and chimerism are both very rare events in KSHV, presumably because of preferential familial transmission and low horizontal transmission rates, which have in general maintained high levels of linkage between multiple loci across the genome. In contrast, similar multisite analysis of variability and cluster patterns across the HCMV genome has revealed much higher levels of genetic scrambling, with a nearly total loss of subtype linkage between nonadjacent variable loci, apparently resulting from rampant intratypic recombination and extensive penetrance of subtypes across ethnic and geographic boundaries (Zong, J-C et al., unpublished data).

2

KSHV Genetic Variability

2.1

Early Studies of VIP Hypervariability Patterns

Two very different types of variability occur within the VIP (K1) and TMP (K15) proteins encoded at the extreme LHS and RHS, respectively, of the KSHV genome. VIP is a 289-amino acid lytic cycle membrane-associated and ITAM-containing IgG family glycoprotein that displays up to 30% amino acid differences between isolates and clusters into five major subtypes (A, B, C, D, and E) plus numerous minor variants that correlate well with ethnic and geographic ancestry in different human host populations (Biggar et al. 2000; Cook et al. 1999; Fouchard et al. 2000; Hayward 1999; Kadyrova et al. 2003; Lacoste et al. 2000a, 2000b; Lagunoff and Ganem 1997; Meng et al. 2001, 1999; Nicholas et al. 1998; Treurnicht et al. 2002; Whitby et al. 2004; Zhang et al. 2001; Zong et al. 1999, 2002). VIP is unique to KSHV and functions as a Tyr-kinase signaling protein that has antiapoptotic properties, can either mimic or block B-cell receptor activation, and appears likely to play an important role in initial establishment or control of latency (Damania and Jung 2001; Lee et al. 2003, 2005; Wang et al. 2004).

Variability within the VIP gene displays very high nonsynonymous rates, with up to 85% of nucleotide changes creating amino acid changes, implying that some as yet unknown but powerful biological selection process has been involved. Furthermore, this variability can be divided into three distinct levels. First, the oldest level occurs predominantly between subtypes and is distributed relatively evenly across the whole gene. Second, a more recent level is observed even within subtypes but is largely limited to two 40-amino acid blocks referred to as VR1 and VR2. The third level is found even within specific variants but is restricted to a 20-amino acid Cys bridge bounded hypervariable domain (VR*) that resembles the VR3 loop in the HIV ENV protein and appears to correlate with strong T-cell epitopes (Stebbing et al. 2003; Zong et al. 1999, 2002). However, unlike the generation of rapidly evolving pseudospecies of HIV or HCV genomes within a single patient, we have never detected any convincing evidence for multiple variants of KSHV VIP either between multiple KS lesions and PMBC samples or within multiple PEL cell lines derived from a single patient. We have concluded that our evidence for highly ethnically restricted clusters of closely related VIP proteins indicates that (unlike most other herpesviruses) KSHV transmission is predominantly familial, with very low rates of horizontal or multiple infections.

We previously interpreted the patterns of distribution of specific VIP clusters as indicating that the principal VIP subtypes arose during the migration

of modern humans out of east Africa first into sub-Saharan Africa (B branch), then into south Asia, Australia, and the Pacific Rim (D/E branch), and finally into the Middle East, Europe, and north Asia (A/C branch), with very little subsequent remixing (Hayward 1999; Zong et al. 1999, 2002). The divergence of the B branch from the progenitor of all the other branches is judged to have occurred approximately 100,000 years ago, with the split of D/E from A/C occurring close to 70,000 years ago. The current estimated times of divergence of D from E and of A from C are 50,000 and 35,000 years ago, respectively, based on both the overall evidence about human migrations and the lengths of the branches in the VIP phylogenetic trees. Within the A and C branches, individual variant clades (A1 to A10 and C1 to C7) probably arose between 10,000 and 12,000 years ago as human populations expanded out from a limited number of Eurasian Ice Age refuges.

2.2

TMP Allelic Variability and Chimeric Genes

TMP(K15), also known as LAMP, is a 500-amino acid latent-state membrane protein with twelve transmembrane domains encoded by an eight exon spliced mRNA and related to the LMP2 latency protein of EBV. TMP is also a Tyrosine kinase signaling protein, although it lacks the ITAM motifs present in both KSHV VIP and EBV LMP2, and by analogy with LMP2 probably contributes to maintenance of the latent state (Brinkmann et al. 2003; Sharp et al. 2002). TMP genes from different KSHV isolates have been described to fall into two alternative allelic subtypes, referred to as P (prototype) and M (minor), that have diverged by 70% at the amino acid level but otherwise show little variability (Choi et al. 2000; Glenn et al. 1999; Hayward 1999; Kakoola et al. 2001; Nicholas et al. 1998; Poole et al. 1999). It is important to appreciate that only a very small segment at the extreme RHS of those genomes that carry the TMP-M allele show this high level of divergence: In fact, it is limited to just the TMP gene itself. However, the patterns of divergence in the region adjacent to the TMP gene (ORF75E and UPS75) are very interesting and complex and provide critical insights and information about the origin and history of the TMP-M allele. Furthermore, about 20% of all human KSHV genomes examined have the TMP-M allele, but they can be found associated with all three of the major branches of VIP genes (A/C, B, or D/E). In essence, there are two overall classes of KSHV genomes, the predominant P class that are considered to have the modern P pattern throughout their length and a second less prevalent class that are chimeric hybrids of P genomes with just a small highly diverged M allele segment at the RHS.

We originally proposed that the TMP-M alleles are of nonhuman origin and are carried only in relatively rare genomes that consist of a predominantly modern human KSHV genome joined to an exotic Old World primate-derived TMP gene at the RHS (Poole et al. 1999). These chimeras were evidently generated by a two-step recombination process, which we suggested involved an initial chimera that was created by a single original cross-species recombination event from an exotic highly diverged primate KSHV-like virus, followed by a second event from a much less diverged premodern humanoid lineage virus, such as might be expected to have been present in Neanderthals, for example. However, there is an alternative possibility that the parent M-type virus from which they originated was instead an anciently diverged true second species of human KSHV virus, which has since either become extinct or has not yet been detected as an intact genome within modern humans (Hayward 1999; Zong et al. 2002).

2.3

More Recent Comprehensive Analyses Including the Central Constant Segment

Subsequent to those original reports, we have continued to extend our detailed analysis of KSHV genome variability in three ways. First, we have sequenced a large number of additional VIP genes (now totaling 180), especially from two major new collections in sub-Saharan Africa, including PBMC from 35 KS patients in Uganda and KS biopsies from 20 patients in South Africa. Second, we have sequenced a total of 60 complete TMP genes from a selected subset of the same samples evaluated previously for VIP, as well as from many of the new African samples. Third, we have also carried out PCR sequencing at up to 10 additional internal genomic loci in many of these same genomes (averaging between 60 and 150 samples each).

Interpretation of the results of these new data from all three segments of the genome now permits refinement and expansion of our previous models, leading especially to the concepts of both chimeric herpesvirus evolution and differences in the recent history of the “new” LHS and “old” RHS ends of modern human KSHV genomes. Based on the overall picture that has emerged, we can now classify known KSHV genomes into a model involving “12 principal genotypes” (see Fig. 1), in which we view each genotype class as being composed of variable-length segments with different estimated ages of divergence that correspond to the different degrees of variability or sequence conservation found at the 12 PCR loci studied. Unlike the hypervariable VIP and TMP genes, the level of variation at the 10 internal constant region loci is much smaller (on the order of 1% to 5% nucleotide differences only), and in this case the polymorphisms rarely affect the primary protein structure,

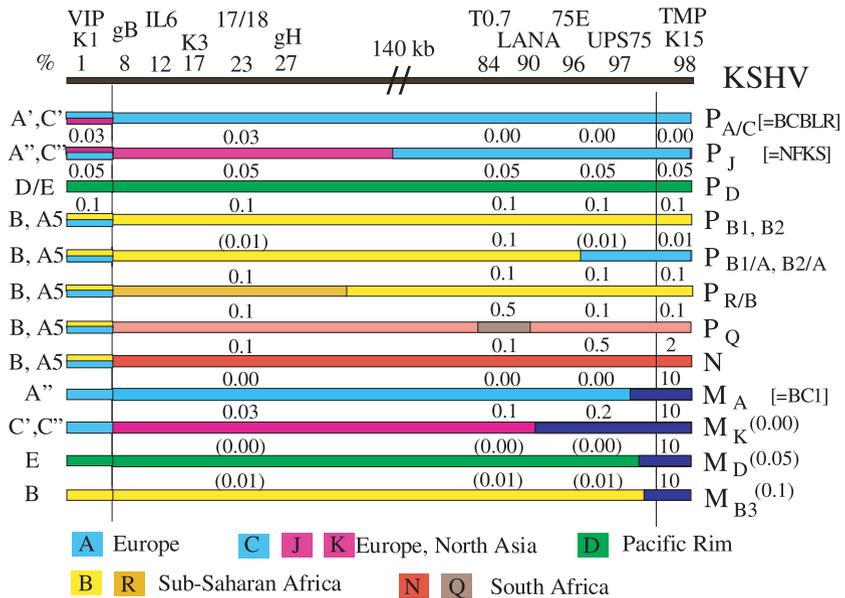


Fig. 1 The 12 major KSHV genotypes model. The diagram illustrates the deduced segmental and chimeric patterns of the 12 principal KSHV genotype structures. The linear genomic positions (%) of the PCR loci used are depicted at the top above the genome lines. For simplicity, the central segments (including the RAP/K8.1 locus at position 37) have been omitted (//). The allelic and subtype descriptions are given to the right, and the nine segment subtype color codes are denoted below the diagram. In addition, the exotic RHS M allele segments are designated by the dark purple bars. The estimated ages of divergence from the prototype Eurasian P genome on the top line are given for each major segment of each genotype in millions of years ago (MYA values)

but nevertheless they still show parallels to the subtype clustering patterns observed in VIP and TMP. Where possible, we have tried to obtain data for the complete VIP gene, not just fragments of it, and to analyze nearly all of the internal loci for each genome to obtain a complete and representative sampling picture across each genome.

3 Phylogenetics of the Hypervariable VIP or K1 Genes

A stylized generic radial phylogenetic tree showing the major branching and cluster patterns and divergence ages of VIP proteins from major human

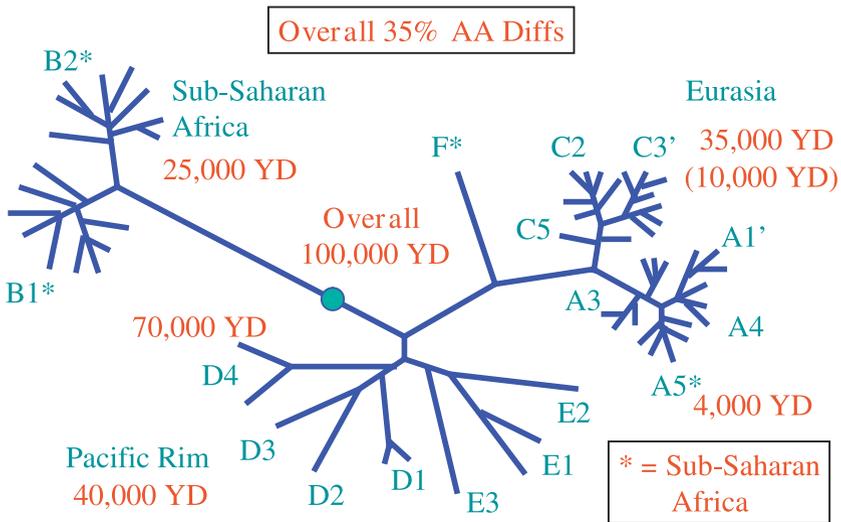
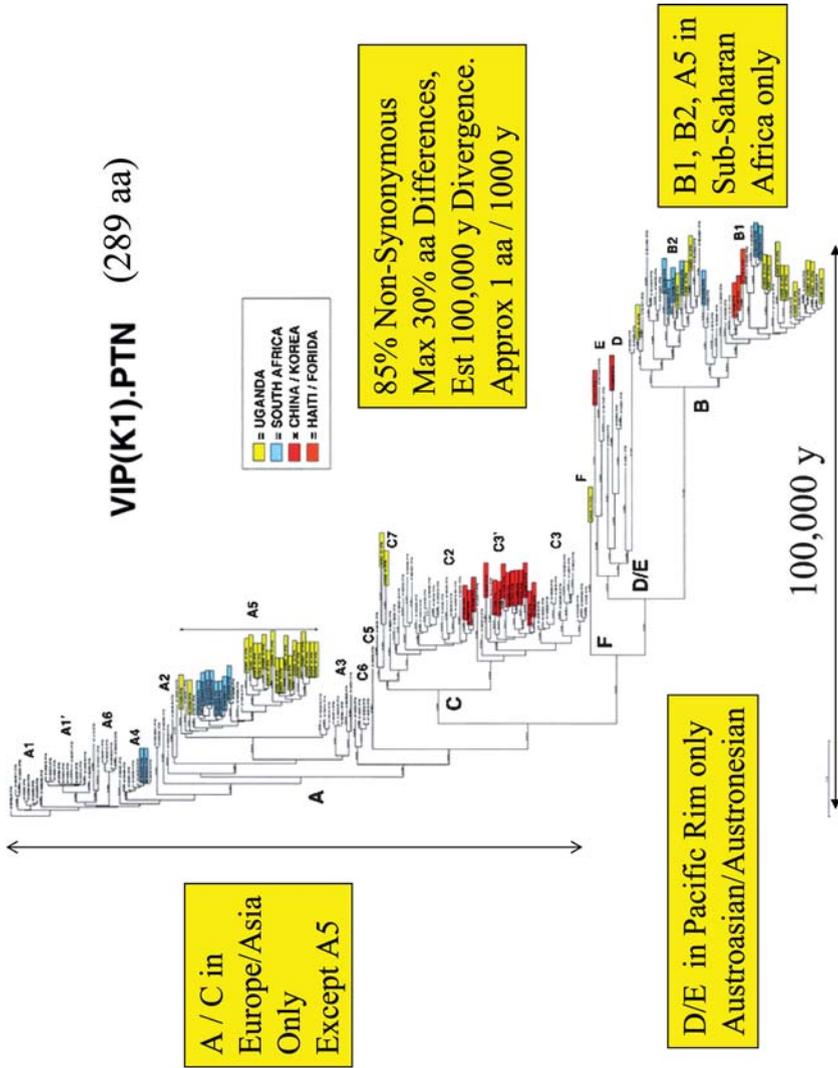


Fig. 2 Overall radial tree branching pattern for the KSHV VIP(K1) hypervariable ITAM membrane signaling protein. The diagram summarizes the major features of clustering and relative distance and branching patterns for samples from Eurasia, the Pacific Rim, and sub-Saharan Africa. The *asterisks* denote the four VIP subtypes found only in sub-Saharan Africa (B1, B2, A5, and F)

Fig. 3 Detailed linear phylogenetic tree for all 180 VIP proteins analyzed in our studies. This tree is based on the complete 289-amino acid VIP/K1 ORF. The entire tree is judged to contain only “modern” VIP subtypes, representing no more than 100,000 years of divergence. Overall, the VIP protein displays 30% amino acid variability, with 85% of the nucleotide polymorphisms being nonsynonymous. This gives an estimated rate of change of approximately one amino acid per 1,000 years. *Color patterns* highlight the samples from Uganda (*yellow*), South Africa (*blue*), Chinese Taiwan and Korea (*pink*), and American samples with African connections (*green*). The two Taiwan samples in the D and E branches were from aboriginal Hwalian samples. HKS22 from Uganda represents the sole example of an F subtype VIP gene found. In general, the A and C subtypes are found only in European and Asian samples, whereas B subtypes are found only in sub-Saharan African populations, and D and E subtypes are found only in Austronesian/Austroasian branch populations including Polynesians and Amerindians

population groups is presented in Fig. 2. The actual linear phylogenetic VIP protein tree for all 180 KSHV samples that we have sequenced plus a few representative samples from other studies is shown in Fig. 3, with key ethnic subgroup collections indicated in the color key.



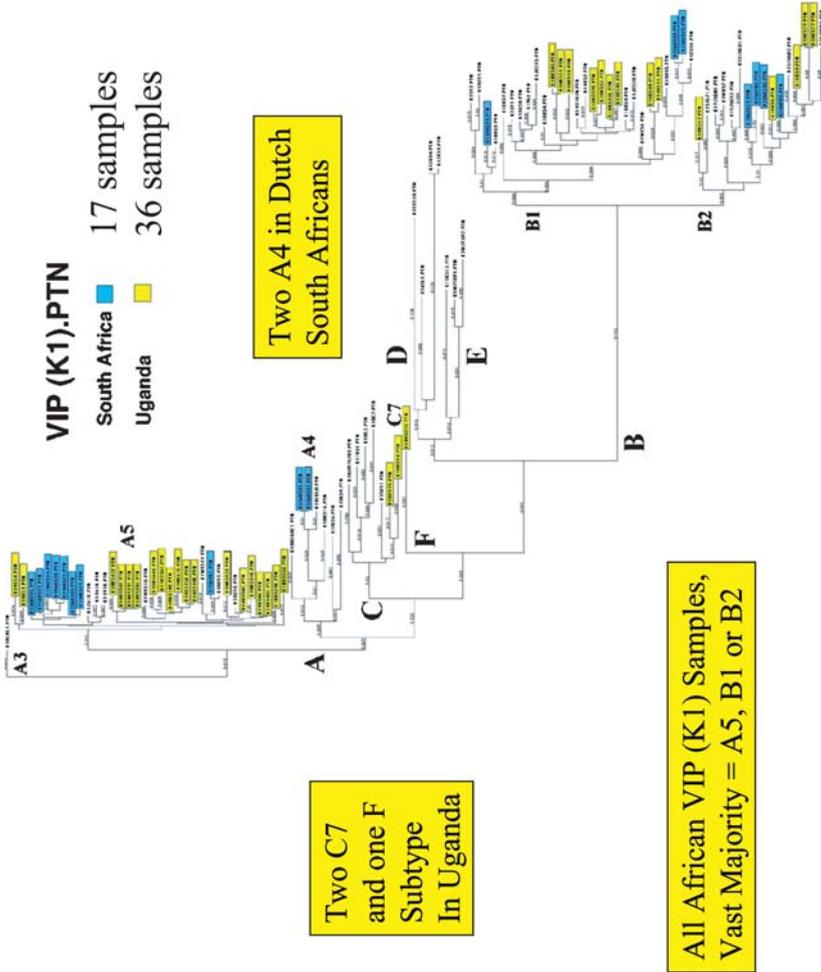
3.1

Age and Origin of B-Subtype VIP Genes in Sub-Saharan Africa

In our original analysis of 60 complete VIP genes from around the world (Zong et al.), eight of nine samples from sub-Saharan Africa were B subtypes, including our original B prototype (431K), and one was the prototype A5 variant (OKS3). Only five other examples of B-subtype VIP genes were found at that time, but four of these were also from patients of African origin, including ZKS6 and JKS20 from African emigrants to New Zealand and the United States, respectively, JKS15 from an African American patient in Baltimore, and OKS7 from a Haitian patient in Miami, Florida. OKS8, which closely resembled OKS7, came from a Mexican woman with AIDS in Miami, and it remains the only exception that we have found of a B-subtype VIP gene without an obvious direct connection to Africa. A total of 30 additional complete B-subtype VIP genes have now been sequenced and the overall results are shown in the partial VIP phylogenetic tree given in Fig. 4. All but one of the B-subtype genomes came from sub-Saharan Africa (either Uganda or South Africa), with the exception being the VG1 PEL sample from Haiti. Data for an incomplete internal segment of the VIP genes from the same 23 HKS samples described here were also presented recently in an analysis of just the VIP genes from patients with different tribal affiliations from Uganda (Kajumbula et al. 2006).

Overall, the 39 B-subtype VIP proteins that we have analyzed, although all differing from the A/C Eurasian subtype by 30%, show only moderate intratypic variations (totaling 8% at the amino acid level), and they cluster into two very distinctive major branches that we refer to as B1 or B2 variants. 431K is designated as our prototype for the B1 VIP gene, and SAPB3 is designated as our prototype B2 VIP gene. There were 13 B1s and no B2s among our original set of 14 examples of African VIP genes, which included five of five from Gambia, three of four from Tanzania, two from Uganda, and three from USA or Haiti (Zong et al., 1999). However, we have now found that four of eight Bs from South Africa and five of 15 Bs from Uganda proved to be B2 variants rather than B1 variants. Several of the new African VIP genes display novel in-frame deletions within the VR1 or VR2 regions. From the analysis of the partial Uganda VIP genes, the possibility that HKS9 and HKS27 represent a distinct third B3 subgroup was suggested in our earlier analysis (Kajumbula et al. 2006), and this remains plausible for the complete genes, including the South African samples in our larger analysis.

Fig. 4 Detailed linear phylogenetic tree of the Ugandan and South African VIP proteins. The vast majority of all sub-Saharan African samples are B1, B2, and A5 subtypes. The 36 complete VIP proteins from Uganda (PBMC from KS patients) are shown in *yellow*, and the 17 complete VIP proteins from South Africa (KS biopsies) are indicated in *blue*. The diagram illustrates the relatively wide divergence of B1 and B2 branches compared to the very narrow divergence of the A5 clade. The relative branching positions of the five anomalous samples (two A4 from patients of Dutch European descent in South Africa and the two C7 and one F subtype from Uganda) are also indicated



3.2

The African A5 Cluster of VIP Genes

In addition to the characteristic African B-subtype VIP genes, we have also now detected 34 more of the A5 subtype, plus one novel F- and two novel C7-subtype VIP genes from Uganda and South Africa (Fig. 4). In addition, two A4 examples from South Africa represent the only classic European variants that we have found in Africa, but these are the exceptions that prove the rule, because both came from the only known Caucasian patients among the group, who were both South African men of Dutch descent. In contrast, the rare prototype examples of VIP-F (HKS22) and the C7 subtypes (HKS35, HKS54) from Uganda all appear to be of genuine endemic African origin. Surprisingly, A5 variants have been detected relatively uniformly throughout Africa, including within a majority of our samples from South Africa as well as many from Uganda.

The 35 A5 VIP genes that we have analyzed all fall into a very tight and narrowly diverged cluster that is quite distinct from the closest European-north African variant clusters A3 and A7. However, we have never found an A5 subtype VIP gene outside of sub-Saharan Africa. Overall, the clustering of A5-subtype VIP proteins is extremely high, with branch lengths on the phylogenetic tree that are very short, being less than 1/5th that of both the B1s and B2s, suggesting a common origin no more than 4,000 years ago, with the B1 versus B2 (Fig. 4) divergence probably occurring about 25,000 years ago.

One other early study reported only A5-subtype VIP genes in PBMC among 15 children with symptomatic fevers in Zambia (Kasolo et al. 1997), but in retrospect it seems extremely unlikely to us that the particular primers used in that study would have been able to detect B-subtype VIP genes. Therefore, the implication that A5s may predominate in currently circulating viruses associated with lymphadenopathy and febrile illnesses in children, rather than in adult KS (although plausible), needs to be re-evaluated. The A5 VIP proteins are clearly much more similar to Eurasian VIP genes than to the B-subtype VIP genes, and they must have had a single-source introduction from Europe or the Middle East presumably via North Africa (backwards flow). The A5s evidently have been (and presumably continue to be) spreading rapidly across and throughout sub-Saharan Africa over a time frame that appears to coincide with the Bantu expansion from western Africa over the past 4,000 years. It is also important to emphasize that all KSHV genome samples that we have studied that have A5-subtype VIP genes are recombinant chimeras in which only a very small LHS segment is A-like, and the remainder of their genomes consist of typical distinctive sub-Saharan B, Q, R, or N subtypes (Zong et al. 2002) (Zong, J-C. et al, manuscript in preparation).

3.3

Source and Origin of the Novel Ugandan VIP-F and VIP-C7 Genes

Among the total of 69 complete VIP genes that we have studied with clear sub-Saharan African origins, only three, namely HKS35 (C7), HKS54 (C7), and HKS22 (F), which are all from Uganda, do not fit into the B1, B2, or A5 patterns described above. HKS35 and HKS54 cluster together between the two major groups of subtype-C VIP proteins, namely, C''(C3) and C'(C1/C2), and are referred to as the first examples known of C7 variants. These are closest to the C4 and C5 variants from Saudi Arabia (with an estimated divergence from other Cs approximately 15,000 to 20,000 years ago). In contrast, HKS22 lies between the A/C and D/E branches on the VIP protein phylogenetic trees (Figs. 2–4) and is the only sample that we have ever detected that falls outside of the three major branches. It is therefore tentatively referred to as an F-subtype VIP gene, suggesting divergence from the other main branches approximately 50,000 years ago. However, the C terminus of HKS22 is very similar to the C7 pattern, including the diagnostic five-amino acid deletion common to all Cs, and this sample clearly represents a complex F/C intragenic recombinant. In addition to HKS22, there are several other samples known (all from central Africa) that also do not fit readily into any of the currently defined A to E classification patterns. These include SAN2 (Whitby et al. 2004) and UGD23 (Kakoola et al. 2001), plus 43/Ber and 8/Dem (Lacoste et al. 2000b). Although they do differ very significantly from one another, we suggest that these, together with HKS22, are all members of a new fourth main branch that also splits into two subgroups designated F and G.

Finally, there is the question of the source or origin of these rare C7, F, and G VIP variants. In fact, all three genomes containing C7- or F-subtype VIP genes are extremely unusual at all loci tested, being regarded as novel intermediate F or G subtypes at several internal constant region loci (see Fig. 8; Zong, J-C et al., unpublished data) and either having unusual divergent B4- and B5-subtype ORF75 and TMP genes (HKS54 and HKS22) or, in the case of HKS35, having N-subtype ORF75 and TMP genes. We presume that the VIP C7 genes were relatively recently reintroduced into sub-Saharan Africa from the Middle East or Europe, whereas the novel F (and G) subtypes probably represent one or more genuine independent branches that may either never have left Africa or more likely returned into Africa from an early Middle Eastern branch that diverged after the south Asia/Pacific Rim branch but before the Eurasian A/C branch.

3.4

A Novel E-Subtype VIP Gene from a Taiwan Aborigine

Several rare KSHV VIP protein sequences have been described from indigenous populations around the Pacific Rim. Although diverged from one another by up to 24%, these nevertheless cluster into either of two distinctive D and E branches within a single D/E superbranch that is intermediate between the Eurasian A/C and African B branches (Figs. 1–3). Examples of D-subtype VIP genes include one (D1) from a Taiwan Hwalian aborigine (Zong et al. 1999), two (D2) from Pacific Island Polynesians seen in New Zealand (Zong et al. 1999), one (D3) from Australia that is presumed to be of aboriginal origin (Meng et al. 1999), and three (D4) of presumed Ainu origin from Hokaido in Japan (Meng et al. 2001). In addition, two VIP genes from Tupi Amerindians in Brazil were designated as the first examples of a new VIP subtype E1 (Biggar et al. 2000). More recently, Whitby et al. (Whitby et al. 2004) have also described five more partial VIP sequences (E2) from two Ecuadorean Amerindian tribes that branch close to the Tupi samples.

Interestingly, a second Hwalian sample (TKS13) that we have analyzed also clustered with the nearly identical D1 (TKS10)- and D2 (ZKS3)-subtype genomes in both the ORF26 and T0.7 constant region loci (Zong, J.-C. et al., manuscript in preparation) but proved to have a VIP protein that is more similar to the E subtypes than to TKS10 or any other Ds. Therefore, we propose to designate TKS13 as E3 in contrast to the Brazil (E1) and Ecuador (E2) Amerindian subtypes. Furthermore, on the RHS, TKS13 proved to have a TMP-M allele, whereas TKS10 and the two Polynesian samples (ZKS3, ZKS4) have TMP-P alleles (see Fig. 5 and below). Unfortunately, sequence data for the constant region loci and TMP are not available for comparison from any of the other known Pacific Rim KS genomes with D or E VIP genes. Overall, we interpret that these 14 genomes with D/E pattern VIP genes were all derived from human populations that have common ancestors who initially migrated out of Africa approximately 70,000 years ago as a single distinctive south Asian branch, giving rise to all subsequent native Australian, Austroasian, Austronesian, and Polynesian populations, as well as Ainu and Amerindian branches, that is, collectively representing a Pacific Rim branch of modern human KSHV. Furthermore, all seven individual variant lineages within the D and E subtypes exhibit much longer branches than any of the variant clusters within the A, C, or B branches (Figs. 1–3), and therefore we suggest that all of these diverged from one another at least 40,000 years ago.

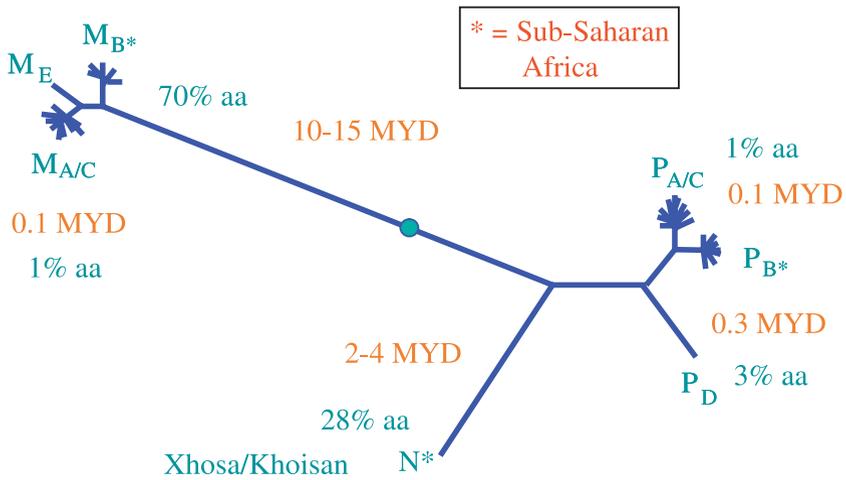


Fig.5 Overall radial tree branching pattern for the KSHV TMP(K15) membrane signaling protein. The diagram summarizes the clustering, relative distance, and branching patterns for samples from Eurasia, the Pacific Rim, and sub-Saharan Africa. Estimated divergence times in millions of years (MYD) are given in *red*, and the overall % amino acid difference levels are shown in *blue*

4

Variable Sequence Loci within the Constant Region

To contrast with the hypervariable LHS and RHS termini of the KSHV genome, we refer to the remainder of the genome as the constant region. However, there are patches of low-level nucleotide variability all the way across the genome, and we have selected known sites of variability within the constant region as likely to be the most informative. The specific internal loci chosen for PCR sequencing analysis range in size from 640 bp up to 1,900 bp and include similar or extended versions of four previously utilized loci (ORF26E, T0.7, ORF75E, and UPS75) as well as five new loci encompassing segments of the vIL6, vMIR2/K3, ORF18/19, RAP/K8.1, and LANA1 genes. Some data have also been obtained for the UL22(gB) locus, but other potential loci such as the ORF4, vIRF1, vCYC, and vGPCR genes showed much less variability still. All of these nine chosen PCR loci together represent a sampling of 7,823 nucleotides (or 5.6% of the 137,000-bp unique segment genomes). Across this sampled part of the constant region we have detected a total of 409 chimeric nucleotide positions (5.2%), with the differences between a prototype Eurasian P(A/C) genome and the prototype P(B1) genome totaling 86 nucleotides (1.1%).

4.1

Clustering, Subtyping, and Linkage

We then interpreted these results in terms of clustering and subtyping at each locus, as well as in terms of maximal levels of linkage between loci and minimal inferred chimerism. Based on the concept of prototypic nonchimeric genomes assigned for each major subtype [e.g., BCBL-R as a average typical P(A/C) genotype, 431K as an average typical P(B)-subtype genome, and ASM72 as the most typical prototype P/M chimera], we have been able to define matching linked subtype patterns at all loci with considerable confidence. Major clustered deviations from these prototype subtype patterns at each locus were then assigned additional subtype status and matched between loci, using the assumption of maximal linkage. Deviations from the standard average or commonest linkage patterns are then considered evidence for additional chimeric recombination events.

A major feature of the results at all variable gene loci for KSHV (as well as for HCMV) is that the sequences show a very high degree of clustering, whereby almost all samples fall into one or another of a relatively small number of subtypes at each locus that may differ from each other quite extensively but themselves show very low levels of variability. There are occasional examples of intragenic chimerism with very obvious recombination junctions, but it is quite clear that there is not a continuum of variability or an infinite number of subtypes. However, the level of variability between subtypes can vary significantly between the different loci and the number of distinctive subtype branches found can also vary (especially in HCMV).

4.2

Subtype Distribution Patterns Within the Constant Region

One of the most puzzling aspects of the original VIP gene analysis (Zong et al. 1999), was that although it very neatly matched the expected patterns of strain divergence corresponding to founder effects from the migrations out of Africa, it did not display the anticipated patterns of greater diversity within sub-Saharan Africa than elsewhere that would have been expected based on the overall mitochondrial and Y chromosome phylogenetic trees of modern humans. However, this was not the case for the constant region loci, where much greater and older divergence is indeed found within the samples from sub-Saharan Africa. For example, we now recognize and define nine distinct subtype clusters within the T0.7 locus. With a few key exceptions, three of these are almost exclusively found only within Eurasian KSHV genomes (A/C, J, and K/M), whereas the D/Es are found only in aboriginal Pacific Rim samples and the other five (B, F/G, R, Q, and N) are found exclusively within sub-Saharan

African samples. All other constant region loci are also much more similar to this cluster distribution pattern than they are to the VIP patterns.

A summary of the observed levels of nucleotide divergence between subtypes at each of nine different constant region loci is presented in Table 1. Note that this analysis omits the hypervariable VIP and TMP loci and therefore greatly underestimates the overall level of variability between subtypes. There are three initial points to be made. First, within the constant region sites (especially at the RHS) there is essentially no distinction at all between genomes that have the A- and C-subtype VIP genes, and therefore we refer to them all as A/C. Second, there are nevertheless two other distinct subtypes within Eurasian constant region loci, which we refer to as J and K. Both are intermediately diverged compared to A/C and B, but there is a pattern whereby the “older” J and K constant region subtypes are preferentially linked to the VIP superpatterns referred to as A' and C', whereas the “newer” A/C subtype constant regions are preferentially linked to the A'' and C'' VIP superpattern genes (Zong et al. 2002). Furthermore, essentially only the K subtypes show direct linkage to the RHS TMP-M alleles. Third, it is evident that although the F- and G-subtype constant region genes are generally less diverged from A/C than the B patterns, the novel R, Q, and N subtypes are most often even further diverged from the Eurasian subtype than the typical African B pattern genomes.

4.3

The 12 Major Genotype Patterns in KSHV

Combining the results of our analyses for all three regions (i.e., the LHS VIP gene, the central segment constant region loci, and the RHS TPM alleles), leads to an overall picture that can be summarized in a highly simplified format by dividing the genotypes into 12 very distinctive and representative patterns as shown in Fig. 1. The subtype designations of different domain lengths of each genome are illustrated by the colored bars, with dark purple bars denoting the exotic TMP-M alleles. We envisage each of the different genotypes here as being composed of multiple segments with different ages of divergence. Numerical estimated ages (in millions of years) for each segmental domain are given in terms of the timing of its original divergence from the predominant European A prototype pattern, which is presented on the top line. Overall, there is a dramatic trend from left to right across the genome of finding relatively young segments only at the extreme LHS, toward retention of medium-age to older segments as well in the center, and then with some much older segments included as well toward the extreme RHS of the genome. The major features that contribute to this model of the 12 prototype genomes are described in the sections below.

4.4

Geographic Distribution of Constant Segment Subtypes

It is also dramatically evident that, just as for VIP, there are major differences in the distribution of constant region subtypes between the Eurasian samples (Fig. 6) and those from the Pacific Rim (Fig. 7, upper group) and sub-Saharan Africa (Figs. 7 and 8). In fact, in most cases there are also major differences

KSHV – EURASIAN GENOTYPES

	K1	gB	vIL6	K3	18/19	26E	T0.7	LNA	75E	UPS	K15	
BCBL-R	A1	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	P	
BCP1	*	A1	A/C	A/C	A/C	A/C	A/C		A/C	A/C	P	
BCBL-B		A4	A/C	A/C	A/C	A/C	A/C		A/C	A/C	P	
2x SAKS21		A4	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	P	HOLLAND
EKS1	*	o C2				A/C	A2		A/C	A/C	P	EGYPT
BKS12	*	o C2				A/C	A2	A2	M	M	MX	
BC1		A2	A/C	A/C	A/C	A/C	A2	A2	M	M	MX	
BKS16		A6					A6	A2	M	M	MX	
BC2		C3	A/C	A/C	A/C	J	A/C	A/C	A/C	A/C	P	
JSC1		C3	A/C	A/C	A/C	J	A/C		A/C		P	
BKS15		C3				J	A/C		A/C		P	
2x RGKS3/4	*	C3		K		A/C	A/C		A/C	A/C	P	SICILY
2x BKS3/4	*	C3				J	A/C				P	
ASM-K3		A1	J	A/C		A/C	A/C	A/C	A/C	A/C	P	
OKS13		C3				A/C	A3		M	--	M	MX
SKS3	*	o C2				K	A3		M	M	M	MX
SKS9	*	o C5				K			M			MX
BC3		C3	A/C	J	K	J/K	J	A/C	A/C		P	
ARK1	*	o A7	J/K	J	J		A/C	A/C		A/C	P	ITALY
BCBL1	*	o A3	J/K	J	J	A/C	A3	A3	A3	A3	P	
OKS18	*	o C2					C2			A/C	P	
KSF		C3	J/K	J	K	J/K	A/C	A/C	A/C		P	GERMANY
BKS20	*	o A7					K	A/C		A/C	P	
11x KKS5	*	C3'		J	J/K	K		M	M	M	M	MX
3x KKS6	*	o C2	J/K		K	J/K	K	M	M	M	M	MX
BKS21	*	o C2				J/K		M	M	M	M	MX
BKS13		o C1				K		M	M	M	M	MX
ASM70		o C1	J/K	K	K	J/K	K	M	M	M	M	MX

* = Classical, non HIV associated o = Inner, older A' + C'

Fig. 6 Whole genomic structural summary for 43 Eurasian KSHV samples, including 23 P/M chimeras. Color coding distinguishes the M-associated segments (red) from P(A/C) patterns on the RHS, as well as the intermediate-aged P(J) and P(K) subtype patterns (pink) from the P(A/C) patterns on the LHS. MX indicates the exotic M TMP allele. The chart diagram is based on direct PCR data for up to 10 loci across each genome. Multiple related samples that have the same or very similar patterns are shown together on a single line

KSHV – PACIFIC RIM and SOUTH AFRICAN GENOTYPES

	K1	vIL6	K3	26E	T0.7	LNA	75E	UPS75	K15	
BCBL-R	o A1	A/C	A/C	A/C	A/C	A/C	A/C	A/C	P[A/C]	USA
ASM70	o C1	K	K	K	M	M	M	M	M[A/C]	USA
TKS10	o D1		D	D/E	D/E	D	D	D	P[D]	TAIWAN/H
TKS13	E2			D/E	D/E			E/M	M[E]	TAIWAN/H
2x ZKS3/4	D2			D/E	D/E		D	D		NZ
431K	o B1	B1	B1	B1	B1	B1	B1	B1	P[B]	ZAIRE
3x RKS3	B1	B2	B2	B2	B2	B1	B	B1	P[B]	ZAMBIA
RKS1	B1	Q	Q	B2	B2	B1	B	B1	P[B]	ZAMBIA
O21K	A5	Q	Q/B3	B2	B2	B1	B	B1	P[B]	ZAIRE
RKS2	B1	B2	B2	B2	B1	M'	A[B]	A[B]	P[AB]	ZAMBIA
391K	A5	B1	B1	M'	B1	B2	R/A	A[B]	P[B]	ZAIRE
RKS5	B1			M'	B1	M'	A[B]	A[B]	P[AB]	ZAMBIA
3x VG1	B1	B3	B3	B2	B3'	B2	A[B]	B3/M	M[B]	HAITI/FL
2x SAKS21/31	A4	A/C	A/C	A/C	A/C	A/C	A/C	A/C	P[A/C]	RSA/HOLLAND
SAKS32	B2	R	R	B2	B3	B1	B	B2	P[B]	RSA
SAKS24	A5	Q	Q	Q	B1	A[S]	A[S]	A[S]	P[AS]	RSA
3x SAKS29/33/35	o A5	Q	Q	Q	QX	A[Q]	M[Q]	B[Q]	P[B]	RSA
2x SAKS25/30	o B1'	Q	Q	Q/B	B1'	R	N	N	N	RSA
2x SAKS22/28	A5	N	N	N	B3	B1	B	B2	P[B]	RSA
SAKS23	o A5	N	N	N	N	N	N	N	N	RSA
SAKS26	B2	N	N	N	B1	R	R/M'	M'	M[A]	RSA
SAKS27	B2	N	N	N	B3'	N	M[Q]	B[Q]	P[B]	RSA
ZKS6	B1'		Q		B1'	N				RSA/NZ

o = PROTOTYPES

Fig. 7 Whole genomic structural summary comparing four Pacific Rim KSHV samples (*upper group*) with the 15 South African samples (*lower group*) plus 14 other non-Ugandan samples (*central group*). Color coding distinguishes novel D, E, K, M, M', and Q segments from prototype A/C or B patterns in the *top* and *center* groups, respectively, and distinguishes the A[S], A[Q], Q, R, M, and N segments from prototype A/C or B patterns in the *lower group*. RSA, Republic of South Africa. The three prototype PA/C(BCBL-R), PB(431K), and P/M(ASM70) genotypes are also included

between the subtype patterns found in our South African samples (Fig. 7, lower group) and those found in Uganda (Fig. 8) or other places in central Africa (Fig. 7, center group). In particular, both LHS and RHS N-subtype segments were found almost exclusively in South Africa, and LHS R-subtype segments were found primarily in Uganda, whereas LHS Q patterns were common throughout Africa.

One of the most notable features of sub-Saharan KSHV genomes compared to Eurasian and Pacific Rim genomes is an evidently much greater degree of chimerism within the central constant segment region, with many genomes that fall into just one of the “African” classes, in fact differing extensively

KSHV – UGANDAN GENOTYPES

	K1	vIL6	K3	26	T0.7	LNA	75E	UPS75	K15	
HKS22	F	R/B	B4/Q	K/R	A	A[Q]	F	B7	P[B]	GISU
HKS40	B1	F	K	J	B1	M'	A[B]	A[B]	P[AB]	G
HKS21	B1	F	K	J	B1'	M'	A[B]	A[B]	P[AB]	G
HKS54	C7	F'	K'	A/C	B4	A[B]	G	B5	P[B]	NYANKOLE
HKS35	C7	Q	Q	R	M	R	N	N	N	KAKWA (N)
HKS17	B2		Q		A2	R	H		P[AB]	LUO (N)
HKS41	B1	R	R	B2	B2	A[Q]	F	B3/M	M[B]	SOGA
HKS49	B1	B1	B1	B1	C5	A[Q]	F	B3/M	M[B]	NKOLE
HKS11	B1	B1	B1	B1	C4	A[Q]	F	B3/M	M[B]	NYORA
HKS56	A5	Q	Q	R	B1	R*	A/M	B6/M	M[A]	KIGA
HKS19	B1	R	R	B2	S2	B1	B	B1	P[B]	TORO
HKS27	B2	R	R	B2	B2	B1	B	B1	P[B]	G
HKS9	B2	R	R	B2	B1'	R	B	B1	P[B]	G
2x HKS20,47	A5	Q	R	B2	B2	B1	B	B1	P[B]	G,G
HKS23	A5	Q/R	R	B2	B2'	B1	B	B1	P[B]	G
HKS43	A5	Q	B1	R		B1	B			G
HKS50	A5	Q	R	B2	B2	B1	B	B1	P[B]	SOGA
HKS61	A5	Q	Q	B2	B2	R	B	B1	P[B]	G
2x HKS13,58	A5	Q	Q	B2	B2	B1	B	B1	P[B]	G, G
3x HKS24,34,52	A5	Q	Q	B2	B2	B1	B	B1	P[B]	TORO, LANGO(N),SOGA
2x HKS7,18	A5	Q	Q	R	B1	R	R/A	A[B]	P[AB]	G, NKOLE
2x HKS10,15	A5	Q	Q	R	B1	R'	R/A	A[B]	P[AB]	KIGA
HKS29	B1	Q	Q	R	B1	A[Q]	R/A	A[B]	P[AB]	G
HKS32	B1	Q	Q	R	B1	R	R/A	A[B]	P[AB]	G
HKS33	B1*	Q	Q	R	B1	R	R/A	A[B]	P[AB]	ALUR (N)
2x HKS36,60	A5	Q	Q	B2	B1	R	R/A	A[B]	P[AB]	G, G
HKS59	B1	Q	Q	B2	B1	R'	R/A	A[B]	P[AB]	G

G=GANDA

Fig. 8 Whole genomic structural summary for 34 Ugandan KSHV samples. Color coding distinguishes the segments designated as subtype F, G, H, J, K, Q, R, M', and N from the commonest prototype sub-Saharan African B subtype pattern. Eurasian-like segments designated A, A[B], A[Q], and C are shown in yellow. M-allele and M-associated RHS segments are given in red. Other more complex chimeric segments are also indicated (see HKS22 especially as the most extreme example). Tribal affiliations are also listed on the RHS (Kajumbula et al. 2006). These genotypes are clustered into three distinct categories in the upper, central, and lower groupings, with the latter two being commonest within the Ganda tribe (G)

in the size, patterns and complexity of internal chimeric segments that mix Q, N, F, G, R, or even M' loci into otherwise B genome backgrounds, for example. This is particularly common within a subset of Ugandan genomes derived from non-Ganda tribal sources (Fig 8, upper group). In contrast, the genomes from the Ganda tribe, the predominant Bantu group in Uganda, were of two major types, either those with mostly the common B-subtype patterns throughout except for R or Q at the LHS (Fig. 8, center group) or those with a more complex Q, B, R/A[B] chimeric pattern (Fig. 8, lower group).

5

The TMP-P and TMP-M Alleles and Adjacent Chimeric Regions

Variability in the KSHV TMP(K15) membrane protein mapping at the extreme RHS of the genome represents an entirely different situation than that for VIP. There are huge amino acid variations found between the P and M alleles, but these are not directly associated with the ethnic and geographic diasporas of modern humans. Rather, each allele itself displays the migration out of Africa patterns at a low variability level, and the differences between the P and M alleles (plus now a third newly recognized N allele as well) represent instead a vastly older evolutionary divergence in a gene that is not itself hypervariable like VIP but evidently evolves at the same slower rate as a typical constant region gene. Our reasons for reaching this conclusion are outlined below, and the current picture of TMP branching and divergence levels is summarized in Fig. 6 and Table 2.

5.1

Relative Lack of Variation Within UPS75 and the TMP Coding Regions of the Eurasian Subtypes of the TMP-P and TMP-M Alleles

Although the 15% differences between A and C VIP subtypes probably arose when modern humans first migrated as two major branches into Europe and

Table 2 Nucleotide differences and estimated divergence rates in TMP alleles

A. Other TMP-M Variants compared to the prototype TMP-M strain (HBL6/BC1)					
Name	Total Nucl diffs (2,100)	Exon Nucl diffs (1,500)	Intron Nucl diffs (605)	Total Amino acid diffs (500)	Est. years of divergence
VG1(B1)	27 (1.3%)	16 (1.0%)	10 (1.6%)	10 (2.0%)	100,000
HKS49(B2)	29 (1.4%)	18 (1.1%)	11 (1.8%)	12 (2.4%)	110,000
TKS13(E)	17 (0.85%)	8 (0.55%)	9 (1.5%)	6 (1.2%)	60,000
B. Other TMP-P variants and TMP-N compared to the prototype TMP-P strain (BCBL-R)					
Name	Total Nucl diffs (2,080)	Exon Nucl diffs (1,470)	Intron Nucl diffs (600)	Total Amino acid diffs (489)	Est. years of divergence
431 K(B)	19 (0.95%)	13 (0.9%)	6 (1.0%) ⁺	7 (1.4%)	100,000
TKS10(D)	47 (2.3%)	33 (3.3%)	14 (2.3%) ⁺	25 (5.1%)	250,000
SAKS23(N)	425 (21.3%)	287 (19%)	138 (23%)*	140 (28%)	2,000,000

* Plus several non-contiguous blocks; + 10-bp insert

northwest Asia about 35,000 years ago, the 6% to 9% amino acid variations between and among individual variants of the subtype A and subtype C VIP branch has clearly arisen much more recently, probably within the past 10,000 to 12,000 years since expansion out of Ice Age refuges. In contrast, the rate of change within the TMP gene over this same time period has evidently been very slow and certainly is no greater than that in other typical constant region genes (Zong et al. 2002) (Zong, J.C. and Hayward, G.S., unpublished data). For example, there are very few differences at all (a total of only 8 nucleotide changes over 2500 bp) among nine representative TMP-P genes that we have sequenced from both European and Middle Eastern sources that are associated with a variety of A- or C-subtype VIP genes (Fig. 6). This group also includes the European GK18 sample described by Glenn et al. (Glenn et al. 1999). Similarly, across an 850-bp segment of the adjacent ORF75 gene (UPS75 locus) from a total of 17 genomes from Eurasian sources with TMP-P alleles, there were again no more than three total nucleotide changes detected, irrespective of what variant of either A- or C- subtype VIP genes were present (all those shown as P in Fig. 6). Similarly, among African UPS75 genes there was a surprisingly large subgroup of 22 examples with an A/C-like pattern, referred to as the A[B] subgroup (Figs. 7 and 8). Most of these display just a single distinctive additional nucleotide change from the Eurasian A/C pattern here, although at least nine have a common chimeric junction linking them to the African R subtype at the adjacent ORF75E locus.

In contrast, over 30% of all Eurasian KSHV samples examined, including 15 of 16 from Taiwan Chinese and South Korean KS patients, have the hugely different M-allele version of TMP (labeled M[X] and shown in red in Fig. 6), which is 70% diverged from the P-allele at the amino acid level (Poole et al. 1999; Zong et al. 2002). However, once again, sequencing of the complete coding regions plus adjacent 5'- and 3'-flanking areas from five TMP-M genes from both European and Asian sources showed no nucleotide differences at all over nearly 2,500 bp relative to the prototype BC1/HBL6 TMP-M gene. Similarly, within the nearby conserved UPS75 K/M locus (between positions 840 and 1300), 13 Eurasian genomes with associated TMP-M alleles displayed 16 common nucleotide differences (3%) from the TMP-P-associated UPS75 (A/C) prototype but again contained a total of only one variable position among them. Two other subtype K/M UPS75 genes derived from South Africa (SAKS26, FTKS2) were also identical to one another here but showed three differences from the Eurasian versions (Fig. 8 and see below).

5.2

Distinctive African Versions of TMP-P

Despite the nearly total lack of variation within and between A and C subtypes of both the Eurasian TMP-P and TMP-M alleles, there are consistent patterns of nucleotide differences within both the TMP-P and TMP-M allele versions that originate from sub-Saharan Africa and from south Asia/Pacific Rim sources. Our prototype African versions of TMP-P (431K) and TMP-M (VG1) were sequenced across the complete TMP coding and flanking region (2,500 bp) and proved to display a total of 18 and 29 nucleotide changes, respectively (representing 0.8% and 1.2%), compared to the prototype Eurasian TMP-P (C282) and TMP-M (BC1/HBL6) allele genes. Overall, with the exception of the few novel N-allele forms (see below), all 40 sub-Saharan African TMP-P allele genes examined proved to be either typical B subtypes similar to 431K (22 examples) or typical A/C-like Eurasian subtypes similar to BCBL-R (18 examples). However, there was considerably more sporadic variation among the African B-subtype samples (e.g., 48 variable positions over 2,500 bp) compared to both the African A/C subtypes of TMP-P with only eleven, or to only six across all eight tested Eurasian A/C subtype versions of TMP-P. Notably, all African A/C-like TMP-P genes (and the Saudi Arabian sample SKS1) have one common nucleotide change (431T) compared to all of the Eurasian versions, and therefore we shall refer to the African A/C-like group as the P[A/B1] subtype (Fig. 7).

Among the 23 examples sequenced of the African B subgroup of TMP-P allele genes (P[B] in Figs. 7 and 8), there were nine changes common to all of them. In addition, the predominant B1 subgroup (13 examples) all have six more common changes, whereas the B2 to B6 subgroups, which include eight of the nine examples from South Africa, lack those changes and are more variable. The six previously sequenced African TMP alleles (Kakoola et al. 2001) include three of our B1-subtype (Ugd2, Ugd12, Ugd19) and two of the A[B2] or A[B3] subset TMP-P alleles (Ugd4 and Ugd23) plus one B-subtype TMP-M allele (Ugd10) from Uganda.

5.3

Distinctive African Versions of TMP-M

Similarly, the seven sub-Saharan African TMP-M alleles that we have sequenced all proved to cluster into one of two groups, either those with typical B-subtype patterns similar to VG1 and Ug10 (5 examples) or those with typical Eurasian A/C-like patterns (2 examples). Therefore, although a subset of Eurasian TMP-P and TMP-M genes (like the A5 VIP genes) have seemingly returned from Eurasia to Africa, the majority of both are distinctive African

B subtypes that have not been found outside of either sub-Saharan Africa or related populations in America (e.g., Haiti and French Guyana). Interestingly, within African genomes there is little evidence for any linkage between VIP subtype genes (A5, B1, or B2) with any particular subtype of the TMP gene (whether P_{A/C}, P_B, M_{A/C}, or M_B), and examples of all combinations have been found (Figs. 7 and 8). Again, there was considerably more variation among the six known sequenced African TMP-M_B genes (six variable positions in all over 2,500 bp) compared to their invariant Eurasian TMP-M_{A/C} counterparts, and SAKS26 and HKS56 both showed only one nucleotide difference from the five sequenced Eurasian TMP-M_{A/C} genes. However, in addition, SAKS26 displays a 30-bp (10 amino acid) deletion within exon 8 (codon positions 389 to 398), and therefore represents the first and only example that we know about of a naturally deleted form of the TMP protein.

5.4

Distinctive Pacific Rim Versions of Both the TMP-P and TMP-M Alleles

We next considered whether, like the African specific versions of TMP-P and TMP-M, the KSHV genomes from the Pacific Rim branch might also show D/E subtype-specific TMP patterns. Although there were only four examples available in sufficient quantities to work with, namely, the two Hwalian samples from Taiwan of TKS10 and of TKS13 plus the two Polynesian ZKS3 and ZKS4 samples, these did indeed prove to have distinctive TMP-P(D) and TMP-M(E) patterns that are very different from those of any of the Eurasian A/C versions or the African B versions (Figs. 5 and 7). For the TMP-P(D) genes, TKS10, ZKS3, and ZKS4 were nearly identical across the entire 2,080-bp coding segment, but they displayed 43, 47, and 49 nucleotide differences (2.1%) from BCBL-R(A/C) and 33, 37, and 39 nucleotide differences (1.6%) from 431K(B), respectively. All three also included a 27-bp (9 amino acid) insertion after position 258 within exon 5. Similar to the B versions of TMP-P, TKS10, ZKS3, and ZKS4 also include a 10-bp insertion at the beginning of intron 1 compared to all A/C versions of TMP-P, but only TKS10, ZKS3, and ZKS4 also contain a 15-bp insertion in the noncoding region 93 bp upstream from the initiator codon. Out of 84 total variable nucleotide positions, the 431K(B1) and SAKS33(B2) variant TMP-P genes have nine differences from BCBL1(A/C) in common with TKS10(E) and ZKS4(D), but TKS10 and ZKS4 have 44 and 48 additional unique positions, respectively.

The TMP-M gene coding region from the Pacific Rim sample TKS13 shows 16 nucleotide differences from the prototype Eurasian version ASM70 (0.8%) and 22 from the prototype Africa version VG1 (1.1%). Out of a total of 33 variable nucleotide positions, HBL6(A/C) and TKS13(E) have 17 in common,

VG1(B) and TKS13(E) have 10 in common, and TKS13 has five unique changes. At the amino acid level, the 489-amino acid TMP-M proteins from HBL6(A/C) and TKS13(E) have six differences and VG1(B) and TKS13(E) have seven differences, whereas HBL6(A/C) and VG1(B) have 10 differences overall. VG1 and TKS13 have four amino acid differences from HBL6 in common, and TKS13 has two unique changes.

Therefore, the TKS13(E) subtype TMP-M gene parallels the VIP pattern by being intermediate between the HBL6(A/C) and VG(B) versions and more closely related to HBL6(A/C) than to VG1(B). In contrast, the TKS10 D-subtype TMP-P allele is instead much further diverged from BCBL1(A/C) than are the B versions, and it is 431K(B1) and SAKS33(B2) that are intermediate between TKS10(D) and BCBL1(A/C). For the TMP-P samples there are 25 amino acid differences between TKS10(D) and BCBL1(A/C) and 26 between TKS10(D) and 431K(B), compared to just five amino acid differences between BCBL1(A/C) and 431K(B1) or SAKS33(B2). Furthermore, among the TMP-P(D) genes, ZKS3 and ZKS4 differ from TKS10 at six and eight amino acid positions and between themselves at two positions. Therefore, the evolutionary distance of the TKS10/ZKS3/ZKS4(D) forms of the TMP-P protein appear to be between three and four times further diverged from both BCBL(A/C) and 431K(B1) or SAKS33(B2), compared to the distance that the TKS13(E) form of TMP-M has diverged from its BC1/HBL6(A/C) and VG1(B) counterparts.

5.5

Evidence for Three Distinct Chimeric Boundaries in African KSHV Genomes with TMP-M Alleles

We previously described a 500-bp transition region in HBL6 DNA encompassing the 3'-end of the TMP-M(K15) gene, a putative K14.1 gene, and the adjacent M-associated sequences at the 5'-end of the ORF75 gene (Poole et al. 1999). Within this region the level of sequence homology between the HBL6 M-prototype and the BCBL-R P-prototype changes gradually from 98% down to 80% and then abruptly becomes undetectable except for the coding sequences for several conserved SH3 and TRAF motifs in TMP. This clearly represents a complex ancient recombination junction between the modern genome constant region and a TMP-M allele of exotic origin. Our previously described diagnostic triple primer PCR test across the boundary here permits simple PCR product size-based discrimination between the standard P genomes and the less common P/M chimeric genomes (Poole et al. 1999).

Because of the patchwork homology over this region, the RHS of the chimeric domain is also readily detected within the sequence differences

chart for UPS75, one of our previously described constant region PCR loci (Zong et al. 1997). The distinctive exotic M pattern begins at position 1357 in UPS75 and extends rightward. Importantly, all 13 European and Asian M allele genomes analyzed in the UPS75 locus have essentially the same adjacent, M-associated UPS75 subtype K/M sequences on the LHS, which are very distinctive from all A/C-, B-, and D-subtype UPS75 sequences.

In contrast, among the nine African genomes with TMP-M alleles, there are three distinctly different chimeric patterns. Six samples, including all three American or Haitian genomes (VG1, OKS7, and OKS8) (Fig. 7), and three Ugandan genomes (HKS11, HKS41, and HKS49) (Fig. 8), proved to have an identical recombination junction boundary mapping between UPS75 positions 1220 and 1280 and producing a chimeric B3/M pattern. The Ugd10 sequence reported by Kakoola et al. (Kakoola et al., 2001) also has this same B3/M junction. In contrast, HKS56 displays a second different chimeric pattern referred to as B6/M, with a junction between positions 1290 and 1350, whereas in the two B/R/M' chimeras found in SAKS26 (Fig. 7) and FTKS2 (not shown), the UPS75 sequence pattern (referred to as M') is very similar throughout to the standard Eurasian K/M pattern, although it is distinctive at four positions. These latter also have chimeric B to R/M' boundaries further to the left inside the adjacent UPS75E locus.

Consistent with these patterns, all seven B3/M genomes have the distinctive M[B] African B type of TMP-M gene, whereas both HKS56 and SAKS26 have novel M[A] versions of the Eurasian A/C-subtype TMP-M alleles. Therefore, we suggest that the B3/M genomes all have a common origin from a different source of TMP-M than the Eurasian versions, whereas SAKS26 (plus FTKS2) and HKS56 each arose by separate and more recent secondary recombination events with Eurasian-derived TMP-M containing genomes. Whether the African TMP-Ms once had a common origin with their Eurasian counterparts before the divergence of their migrating human hosts 100,000 years ago, or instead represent more modern independent recombination events from two different Neanderthal K/M-like rhadinovirus genomes, for example, remains an interesting speculation.

Finally, both TKS10 and TKS13 also display complex patterns in UPS75 that suggest possible additional chimeric junctions. In fact, all three extensively analyzed D or E genomes (TKS10, TKS13, and ZKS3) contain similar but distinctive K/M-like segments between UPS75 positions 950 and 1150 or 950 and 1130, with TKS13 also having an additional novel upstream region between positions 720 and 950.

6 **The Rare KSHV N Alleles in Southern Africa**

6.1 **Additional N-Subtype Genomes at the UPS75 Locus**

Alagiozoglou et al. (Alagiozoglou et al., 2000) first described another distinctive KSHV subtype within the 850-bp UPS75 locus from among a set of KS lesion DNAs from South African patients seen in Johannesburg. This set of six samples proved to be almost twice as far diverged from the BCBL-R A/C prototype as were even the M allele-associated K/M sequences in UPS75. We initially confirmed and extended these results with two of the same N-subtype samples described by Alagiozoglou et al. (SALN7, SALN8) (Zong et al. 2002) and have subsequently also found five more examples, three from South Africa (SAKS23, SAKS25, SAKS30) and two from Uganda (HKS35, WKS14). The N-subtype sequence for the UPS75 region between positions 540 to 1300 has 42 nucleotide differences (5.5%) from the UPS75 P(A/C) prototype pattern of BCBL-R, although they vary at just one position among themselves. This compares to 16 differences in this region (2.1%) between BCBL-R and the Eurasian M-subtypes such as ASM70, which again show only one variant position among all 15 European, Asian, and African examples analyzed (Poole et al. 1999). By comparison, there are usually a total of only seven to nine nucleotide differences here from BCBL-R in each of the several different variants of African B-subtype UPS75 genes found. Finally, at this locus, the D1 and D2 subtypes found in ZKS3, ZKS4, and TKS10 were identical, but they all differ at 17 positions from BCBL-R, whereas TKS13(E2) is closely related to the Ds but is also more complex (22 differences from BCBL-R). The branching and divergence relationships among various different UPS75 (as well as LANA and ORF-75E) subtypes are illustrated in Fig. 9.

6.2 **Novel N-Allele KSHV TMP Genes from Southern Africa**

Obviously, the N-subtype UPS75 data suggested that the TMP genes from these KSHV samples might be very different from either the B-subtype TMP-P or B-subtype TMP-M alleles. Indeed, this proved to be correct for all four different KS lesions studied (SAKS23, SAKS25, SAKS30, and HKS35) that had N-subtype UPS75 genes and were available for further analysis. Two of these samples (SAKS25 and SAKS30) were derived a year apart from the same patient, but all four proved to be identical over the entire length of their TMP-N genes. Initially, we were unable to detect any PCR products directly with the standard triple primer reaction from this particular subset of

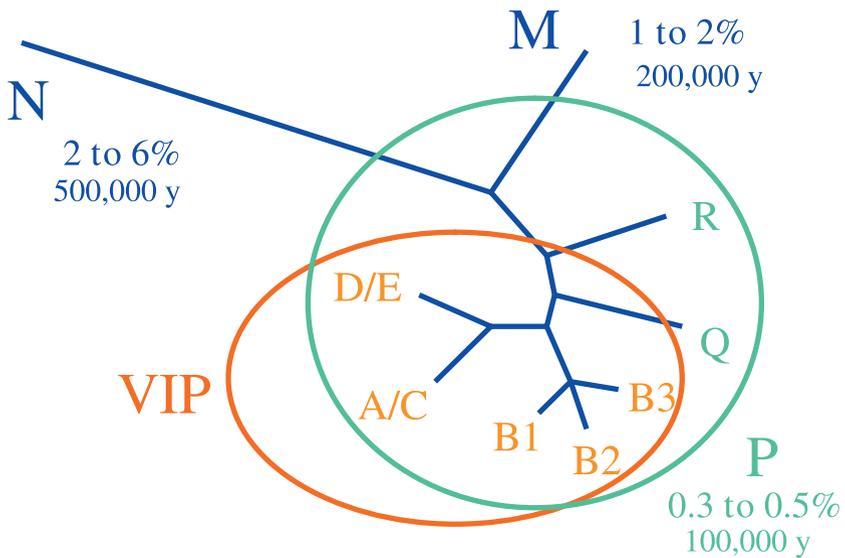


Fig. 9 Summary diagram illustrating the interpreted relative phylogenetic ages for the subtype divergence patterns found within the RHS constant region segments in the LANA, 75E, and UPS75 loci. Note that in addition to the A/C, D/E, B1/B2/B3, Q, and R branches, which are all designated as the P-allele subset (encompassed by the yellow circle); there are also older more diverged M and N allele-associated segments with 1% to 2% (M) and 2% to 6% (N) nucleotide level variation from the PA/C pattern. The red oval encompasses the “modern human” subset of the P-allele VIP gene patterns (A/C, D/E, and B), which are the only subtypes now found at the extreme LHS of KSHV genomes

African samples that were known to have N-subtype UPS75 genes. They were still negative even after hybridization to Southern blots of the PCR DNA with either specific ^{32}P -labeled UPS75-P or ^{32}P -labeled UPS75-M DNA probes (data not shown). However, PCR products were ultimately recovered and sequenced from the SAKS23 prototype-N sample by using two rounds of redundant RHS 5'-primers whose design was based on weak residual DNA homology near the initiator codons of both TMP-P and TMP-M. Subsequently, a series of eight new TMP-N specific primers were generated and used to successfully amplify and sequence the entire 2,500-bp TMP gene and surrounding region from all four previously negative samples suspected of being N subtypes.

This new subset of KSHV TMP genes proved to fall into an intermediate position between the TMP-P and TMP-M allele patterns, although being considerably closer to the TMP-P pattern (Fig. 5). At the nucleotide level, the 2,080-bp SAKS23 coding region DNA including the introns shows 17% differ-

ences from the BCBL-R gene, whereas at the protein level, the novel N-associated TMP gene displays 29% amino acid differences from the prototype TMP-P protein (BCBL-R) and 69% differences from the prototype TMP-M protein (HBL6). Obviously, this is vastly greater than the 0.5% to 2% nucleotide differences for B and D subtypes relative to the prototype A/C subtypes for both TMP-P and TMP-M. Therefore, we consider that TMP-N represents a third distinctive allele with evolutionary hierarchal stature equivalent to that of the TMP-P and TMP-M alleles themselves.

Interestingly, the major structural difference in the TMP-N protein compared to both TMP-P and TMP-M is the insertion of an extra nine amino acids after codon 258 in exon 5, which lies within the fifth extracellular loop. Remarkably, this is also almost exactly at the same location as the novel nine-amino acid insert within the subtype-D TMP-P gene (TKS10, ZKS3, and ZKS4). Furthermore, there is nucleotide homology at 20 out of 27 bp between these two inserts (75% identity), and both encode an NXT/S N-glycosylation motif at exactly the same position. This similarity indicates that the extra domain evidently must represent the primordial situation, with both the M and all other P samples having suffered independent deletions of this NXT/S motif. Each TMP allele has only one other potential NXT/S motif, which maps within extracellular loop 2 for TMP-P and TMP-N and within extracellular loop 3 for TMP-M.

Overall, the 12 transmembrane domains of the three TMP allele proteins show considerably less divergence than the intervening loop domains, and, most importantly, several blocks of amino acids within the cytoplasmic tail of TMP (encoded entirely by exon 8) are virtually completely conserved in all three versions, including regions implicated previously as SH2 or SH3 tyrosine kinase interaction motifs such as YEEVL and YASIL, or the two potential TRAF interaction motifs QSGIS and TQPTD, although a third, SPQPD, is absent in TMP-M (Glenn et al. 1999). Curiously, like both the subtype-B and -D versions of TMP-P, TMP-N also contains a 10-bp insertion at the beginning of intron 1 relative to the TMP-P_{A/C} version, and the sequence here is only 2 bp different from that in the TMP-P_B versions. Two internal 23-bp and 43-bp segments of TMP-N introns 1 and 2 are also very different from the TMP-P versions (14- and 27-bp deletions or mismatches, respectively), and there are also five other 1-bp insertions or deletions spread across introns 4, 5, 6, and 7.

6.3

Extension into the ORF75E Locus Reveals Additional RHS Chimeric Junctions

Data for three of the N-subtype samples (SAKS23, SAKS25, and HKS35) were extended to cover all of the adjacent expanded ORF75E locus (Chang

et al. 1994; Zong et al. 1997), where they differ at 18 polymorphic positions from the BCBL-R (A/C) prototype, although in this segment they also more closely resemble several other patterns, including K/M (4 differences), Q (4 differences), and R (5 differences). In comparison, the D, B, and F patterns for ORF75E differ from A/C at only 6, 9, and 8 positions, respectively. One important additional sample ZKS6 from a Mozambique San bushman patient was also judged to be N subtype at this locus (Fig. 7).

At least three additional chimeric boundaries are detectable within the ORF75E locus in other African genomes. The most common one occurs between positions 159 and 237 in 10 samples (nine from Uganda) that we refer to as the R/A subtype (Fig. 8). All were A[B] in ORF75 and also have common patterns in T0.7 and LANA. Eight were sequenced for TMP, and all were judged to be P[AB1]. Therefore, the size of the RHS Eurasian-derived A/C-like segment present in nearly half of the African chimeric genomes is only 2.6 kb. Interestingly, the second type of chimera here (R/M) also involves almost exactly the same location, but this time joining an R pattern on the left with a K/M pattern on the right in SAKS26 (Fig. 7) and HKS67 (not shown). The third obvious chimeric junction in this region involves an A/C-like pattern to the left of position 528 joined to a B3 pattern on the right in all four American B genomes (JKS15, OKS7 and OKS8, and VG1) (Fig. 7). HKS54 and HKS56 are also very similar, but with slightly different boundaries (A/G and A/B6) (Fig. 8).

7

Differential Evolutionary History of Segments Along the KSHV Genome

7.1

Age of Divergence and Origin of the Three TMP Alleles

In attempting to estimate the age of the evolutionary divergence of TMP-P, TMP-N, and TMP-M alleles from each other, we have based our calculations on the presumption that the B subtypes of both TMP-P and TMP-M (which show between 0.9% and 1.2% nucleotide divergence each from the A/C forms) are judged to have branched away from their common modern human ancestor close to 100,000 years ago. Intriguingly, although divergence of the D/E (Pacific Rim) version of TMP-M in TKS13 (0.85%) fits well with the notion of being 70,000 years separated from the A/C and B versions, the particular TMP-P allele present in TKS10, ZKS3, and ZKS4 (2.3% diverged) is obviously much older than the B version and is projected to have diverged from both the A/C and B versions between 200,000 and 250,000 years ago (Figs. 1 and 5). An

extrapolation for 21.3% nucleotide divergence would suggest an evolutionary distance of 2 to 4 million years for the TMP-N allele from TMP-P, with a further extrapolation to at least 10 to 15 million years for TMP-M from TMP-P. Note that the equivalent gene (R15) in the RRV rhesus rhadinovirus (the RV2 prototype herpesvirus), although showing a strong structural resemblance with 12 transmembrane domains and eight matching coding exons, has no measurable residual amino acid homology to any of these KSHV TMP alleles (Alexander et al. 2000; Searles et al. 1999). The original divergence of rhesus monkeys from great apes and humans is expected to be on the order of 20 to 25 million years ago, but because KSHV fits into the RV1 rather than RV2 lineage of Old World primate rhadinoviruses (Rose 2003; Rose et al. 1997), the RRV difference is probably misleadingly large and that in rhesus RFMHV (an RV1 class) virus would likely provide a better yardstick. In the future, sequence data for the TMP genes from the three distinct KSHV-like viruses known in chimpanzees (Lacoste et al. 2000c), which would be expected to have diverged from the equivalent human and prehuman versions only 5 to 6 million years ago, will go a long way toward clarifying the exact age of divergence of the three TMP alleles found in modern humans.

Whether the events that generated these chimeras occurred between multiple distinct viruses all infecting the same prehuman hosts or instead were derived by recombination with exotic nonhuman but nevertheless “hominid” lineages remains to be elucidated. However, it is very clear from the easily recognized recombination junctions found near the N-terminal regions of ORF75 that both the TMP-N and TMP-M alleles themselves are the only segments of modern-day KSHV genomes that diverged more than 1 million years ago, whereas all of the genomes carrying them that have been detected so far represent chimeras containing these relics of anciently diverged viruses recombined into predominantly modern lineage human KSHV genomes. Nevertheless, the modern KSHV genomes that they originally recombined with (as represented by the adjacent constant regions in the T0.7, LANA, ORF75E, and UPS75 loci, etc.) are also both very distinctive from and significantly older than the standard subtype A/C, B, and D/E level divergence seen in the VIP gene at the LHS and in the central constant segment loci of all other sequenced KSHV genomes. Recombination with, for example, *Homo neanderthalis*, *Homo erectus*, or *Homo robustus* versions of KSHV would be the type of scenario that we envisage may have introduced these small segments of now presumably extinct KSHV-like viruses into chimeric modern human genomes.

However, we cannot exclude the alternative possibility that the complete highly diverged original M genomes that evolved separately in prehumans may still exist in modern *Homo sapiens* but have not yet been detected, perhaps because they are not associated with KS. Consider that (1) chimpanzees are

known to have three distinct rhadinoviruses, two in the RV2 RRV-like KSHV-like lineage and one in the RV1 lineage (Lacoste et al. 2001); (2) all three major branches of modern humans (Eurasian, African, and south Asia-Pacific Rim) have their own distinctive versions of both the hugely diverged TMP-P and TMP-M alleles as either complete P or chimeric P/M KSHV genomes; and (3) at least one of the oldest groups of humans in southern Africa has a representative of yet a third rhadinovirus TMP allele as P/N chimeras. Therefore, the possibility that there are or were three distinct human RV1 lineage rhadinovirus species cannot be ignored.

7.2

Timing of the P/M Chimeric Events

Under either scenario, a question also arises about whether the final recombination event(s) that created the P/M chimeras occurred just once before modern humans came out of Africa or occurred on three separate occasions, once in the African, once in the Pacific Rim, and once in the Eurasian lineage. For the first option to be correct, the small founding human population that initially successfully exited the African continent would have had to have brought two viruses with it, one P and the other P/M, both of which then independently diverged within the three main branches. However, the fact that the three different chimeras each have different junction boundaries clearly favors the alternative scenario in which just a single *Homo sapiens* P genome came out of Africa along with the founding modern humans, but that each of the three branches independently recombined with pre-existing Neanderthal version P/M chimeras that had also diverged into three separate lineages in the three different continental subgroups. In fact, we probably also have to conclude that this type of event occurred at least twice in Africa to create both the relatively common B3/M chimera and the rare B6/M chimera found in HKS56. In addition, a secondary transfer event must have also introduced the Eurasian type M'[A] exotic TMP allele into a B/R genome in Africa (SAKS26 and FTKS2).

We originally favored the scenario that the source (in terms of both time and place) for the origin of the KSHV P/M chimeric genomes was the still overlapping *Homo sapiens* and Neanderthal populations in the Middle East as recently as 25,000 years ago, but the new evidence both here and from Kakoola et al. (Kakoola et al. 2001) for distinct African and now also south Asia-Pacific Rim versions of both the TMP-P and TMP-M alleles instead implies an alternative model in which the original TMP-M allele (probably already in the form of a P/M chimera) must have already been present before the migrations out of Africa. However, whether it was present within modern

Homo sapiens at that time or rather just in an older “humanoid” subgroup is a critical point. If the former, this second type of KSHV virus would have had to be retained in all three branches during the same founder bottleneck events that also led to the divergence of the P versions of the VIP gene into just one main subtype (A/C, B, or D/ E) within each branch. This seems doubtful. Therefore, a more complex version of the original model suggested above, in which all three subsets of TMP-M alleles were instead acquired independently by multiple but rare transfers into modern human P genomes from already existing older and already diverged “Neanderthal” chimeric P/M genomes that occurred after the primary modern *Homo sapiens* migrations out of Africa, seems to be a much more likely prospect. This model also suggests that some form of selective advantage for these particular chimeric recombination products may have been involved (within modern humans at least). Furthermore, the unexpectedly high divergence of the south Asian-Pacific Rim D version of TMP-P, but not of the E version of TMP-M, provides an additional complication to the single parental “out of Africa” concept that needs to be considered in any all encompassing model. However, it could be entirely consistent with divergence of the parental “Neanderthal” P/M genomes into three branches with a temporal pattern different from that for modern humans.

7.3

Source of the Exotic TMP-N Alleles

Presumably the rare P/N genomes would have to represent another example of a similar scenario in Africa, although presumably in this case with a different prehuman KSHV-like virus having a TMP gene allele that had diverged at an intermediate temporal stage between the P and M allele versions. However, it is still necessary to evaluate the full length of the central segments of all of these genomes more thoroughly before any compelling theories can be developed about the source, age, origin, and history of the original parental N-class KSHV genome. Nevertheless, it is probably highly significant that four of the five examples of genomes with both N-subtype TMP alleles and large N-associated adjacent internal segments came from South African sources (and the other one was from Uganda). We have also observed a number of additional South African KS genomes (but rarely Ugandan ones) that have partial N-like constant region segments, but without the associated N-allele TMP genes (Zong J-C and Hayward, G.S., unpublished data). Note that many of these South African patients came from a mining area where there is significant mixed heritage involving Zhosa or Khoisan ancestry. Furthermore, ZKS6, which contains an N-subtype ORF75E gene at least, came from a patient of

known San bushman ancestry who emigrated to South Africa and then to New Zealand from Mozambique. Similarly, Whitby et al. (Whitby et al., 2004) have recently described a San bushman KSHV sample from Botswana (SAN1) that also contains an N-subtype ORF75 gene. These observations strongly hint that both the TMP-N allele and the N-subtype ORF75 gene may be indigenous in Zhosha or Khoisan populations (and may never have left Africa).

7.4

Only Modern Versions of the VIP Genes Have Been Detected

In the case of the VIP genes on the LHS of modern human KSHV genomes, there seems to be a very different evolutionary dynamic in action. Two overall general conclusions can be made. First, only modern P forms of the VIP gene are found, which split relatively recently into the A/C, B, and D/E branches corresponding to the diasporas created by migrations out of East Africa. Second, in sub-Saharan Africa, the VIP genes are essentially randomized and unlinked relative to their associated adjacent constant region subgroup patterns. In distinct contrast to the retention of alternative TMP alleles that are ancient chimeric relics of other viruses on the RHS, no VIP gene subtypes older than the estimated 100,000 years divergence created by the migrations into and out of Africa have been detected. Admittedly, we probably would not be able to detect true M or N versions of the VIP gene directly with the P-subtype primers that we use. However, no KSHV genomes that are detectable with ORF26, T0.7, or UPS75 locus primers (which do amplify all N, M, B, R, and Q constant region variants) have ever proved not to have a detectable linked modern P-subtype VIP gene. Furthermore, all VIP genes found so far fit into the B, A/C, D/E, or F/G branches of the P subtype. Therefore, on the basis of the temporal yardstick of 40,000 years divergence from each other for the known aboriginal Hwalien (Taiwan), Australian, Polynesian, Japanese (Ainu), and Amerindian versions within the D/E branch, we estimate that all known versions of VIP diverged no more than 100,000 years ago. We envisage that the B branch separated from the common ancestor of all of the others approximately 100,000 years ago, then the D/E branch diverged from the A/C and F/G line 70,000 years ago, and finally the A/C branch diverged from the F/G branch about 40,000 to 50,000 years ago. Subsequently, the Eurasian A plus C branches likely diverged from each other no more than 30,000 years ago, with the four main subclusters (C', C'', A', A'') each having branched off 15,000 years ago. Similarly, in sub-Saharan Africa, the original divergence of all known B-subtype VIP genes into the B1 and B2 variants appears to have occurred between 25,000 and 30,000 years ago, but in contrast the entire A5 cluster evidently originated no more than 4,000 years ago.

7.5

Source and Rapid Penetrance of the Anomalous A5 VIP Subtype Within Sub-Saharan Africa

How then did the “European-like” A5 VIP genes become distributed seemingly uniformly throughout central, eastern and southern Africa, as well as become associated almost randomly with genomes that contain B, R, Q, or N constant regions? The interpretation partially depends on the age of divergence of the constant region subtypes themselves, which is very difficult to estimate and clearly varies between gene loci with a pronounced tendency to be greater toward the RHS than on the LHS (Zong, J-C and Hayward, G.S., unpublished data). At nearly all constant region loci, we recognize five distinctive African subtype clusters (referred to as B, R, Q, N, and M') that are at least as far diverged from the A/C cluster as are the Bs, as well as F-, G-, D/E-, J-, and K-subtype clusters that are intermediate between the A/C and B subtypes. Notably, B, R, Q, and N are all unique to sub-Saharan Africa. Consequently, we judge that the R, Q, N, and M subtypes of constant region sequences on the RHS all diverged from a common ancestor with the A/C and B subtypes well before the “out of Africa” migrations, whereas on the LHS some may have diverged contemporaneously with B from A/C. Overall, it is very obvious that there have been no VIP genes yet detected that have the equivalent patterns of divergence expected for the older R, Q, N, and M versions of VIP. Within sub-Saharan Africa, they have all evidently been lost and replaced by relatively modern B1, B2, or A5 VIP genes.

Evidently, a prototype A5 VIP gene must have been originally introduced from Europe or north Africa (probably just once) into presumably a B-subtype genome in the ancestors of the Bantu people at Bok in Nigeria before their expansion across most of the continent beginning about 4,000 years ago. Recombination into the genomes of other ethnic African populations who were displaced or absorbed would account for some spread into the hybrid B/R and B/Q genomes that are now most common among the predominantly Bantu samples that we analyzed in Uganda. However, the Bantu expansion did not proceed beyond the Cook River in South Africa before Europeans arrived several hundred years ago. Yet many of the South African genomes with N-subtype segments, which are presumably of Zhosa or Khoisan origin, also have A5 VIP genes! Furthermore, even the B-subtype VIP genes from South Africa are very little different from those found in Uganda or elsewhere in Africa, in contrast to the often dramatic differences in their constant region subtype patterns (i.e., including Q, R, and N subtypes). We propose that the most likely explanation is a very rapid, and recent, “aggressive” spread through recombination events, which are also presumably connected to some strong

selective advantage in combining a Eurasian A5-subtype VIP gene with otherwise African-specific B-, Q-, R-, or N-subtype constant region genomes. Unfortunately, there is too little divergence in the ORF4 region adjacent to VIP to be able to detect chimeric boundaries there or to judge whether or not the A5/B LHS segment was subsequently introduced into R, Q, and N genomes on just one or on multiple occasions (although we assume the latter).

8

Overall Significance

8.1

Precedents From Other Herpesviruses

There are several obvious precedents for multiple distinct herpesvirus species from the same virus genus coexisting within the same host species. For example, human HSV-1 and HSV-2 have an average protein level divergence of approximately 30%, which is similar to the average protein level divergence of human and chimpanzee CMVs (Davison et al. 2003). HSV-1 and HSV-2 have different preferential sites for establishment of latency in their human hosts and evidently do not successfully recombine *in vivo*, but can do so relatively easily in coinfecting cell cultures under laboratory conditions. Similarly, humans harbor three distinct roseoloviruses with similar but distinct biological properties, HHV6A, HHV6B, and HHV7. The overall divergence level of HHV6 and HHV7 is similar to that between HSV-1 and HSV-2, or between human and chimpanzee CMVs, but although the level of divergence of several genes in HHV6A and HHV6B reaches 15% to 25% at the protein level, most of the rest of their genomes differ by no more than 5% to 10% at the nucleotide level. Again, no known chimeras have been detected, but most individuals harbor HHV7 plus one or both of the HHV6 variants simultaneously.

Finally, the human lymphocryptovirus EBV has genomes that fall into two subtypes, often referred to as EBV-1 and EBV-2 or, preferably, EBV-A and EBV-B. Here the situation more closely resembles that in KSHV, with one gene in particular, EBNA2, occurring as two highly dimorphic alleles known as EBNA2A and EBNA2B that differ by approximately 45% at the amino acid level (Dambaugh et al. 1984), compared to a 55% amino acid divergence for both from the equivalent gene in the baboon lymphocryptovirus, *H. papio* or HPV1 (Ling et al. 1993). Several of the other EBV latency genes also split into A and B subtypes but show only 3% to 5% amino acid divergence, and, except for the somewhat hypervariable LMP1 gene, the differences elsewhere are likely to be no more than a few percent at the nucleotide level.

Therefore, similar to the situation with the KSHV TMP alleles, one of the two forms of human EBV genomes evidently carries a chimeric segment consisting of just the second allelic type of EBNA-2 gene, which must have diverged perhaps 15 to 20 million years ago, and would seem likely to have been derived by an exotic cross-species recombination event into a modern human EBV genome that was slightly different from the current predominant version of human EBV-A. Just as with KSHV, some relatively rare recombinant chimeras containing segments of both EBV-A and EBV-B are known, but the two EBNA2 alleles are both found spread throughout the Eurasian, African, and south Asia-Pacific Rim (Papua New Guinea) branches of modern humans (Aitken et al. 1994). There also appear to be distinctive Eurasian, African, and south Asian-Pacific versions of at least the EBNA2A allele (Shim et al. 1998).

Although it seems extremely unlikely that a complete version of the parental EBV-B-like virus that was the source of the second human EBNA2 allele still exists in modern humans, it does seem plausible that either a complete or a chimeric version of it may have existed in some other now extinct branch of prehumans, such as Neanderthals, and was then transferred probably only once early during the period when modern humans overlapped with them, originally perhaps 100,000 to 200,000 years ago in Africa or even as recently as 25,000 to 30,000 years ago within the Middle East or southern Europe.

8.2

Contribution of Chimeric Fixation to Herpesvirus Genomic Evolution

Irrespective of their precise origins, the presence of these chimeric genomes and alternative alleles of key genes in both KSHV and EBV probably reflect a somewhat unexpected but normal mechanism by which herpesvirus genomes evolve. There are also three radically different alleles of the STP/TIP genes in herpesvirus saimiri (HVS) that may represent a similar situation. Even although herpesviruses are not supposed to successfully cross between species barriers as whole genomes, our data here provide evidence that partial chimeric versions of exotic viral cross-species recombination events probably do occur and do survive successfully at least temporarily (irrespective of the issue of whether a cross-species host transfer is also involved). Presumably, for closely related viral sequences with greater than 85% nucleotide identity, unless there is a selective advantage, the rare novel form will tend to be diluted out and lost by homologous recombination, as we see occurring with the progressively shorter segments of the M-associated K/M constant region sequences found in Eurasian KSHV genomes that retain TMP-M (Kakoola et al. 2001; Poole et al. 1999; Zong et al. 2002). However, for genes that are sufficiently diverged already to be unable to undergo homologous recombina-

nation, that is, greater than 15% differences at the nucleotide level, such as TMP-P, -M, and -N or EBNA2A and 2B, they would not become scrambled or diluted out, especially if there is a positive selective biological effect, and can become fixed in the population, initially just as alternative alleles but eventually perhaps after certain bottlenecks or founder effects narrow down the range of variants in a subpopulation, they could even be destined to take over as the only extant version for a particular virus species.

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Rhesus Monkey Rhadinovirus: A Model for the Study of KSHV

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1	Introduction	44
2	RRV Genome	45
2.1	Genomic Organization	45
2.2	Genomic Structure—Conservation with KSHV	51
3	RRV Transcriptional Program and Characterization of Individual Proteins	52
4	In Vivo and In Vitro Infection with RRV	53
5	Structural Conservation Between RRV and KSHV Capsids	55
6	Structural Conservation Between RRV and KSHV Virions	58
7	The Future of RRV as a Model for the Study of KSHV	65
	References	65

Abstract Rhesus monkey rhadinovirus (RRV) is one of the closest phylogenetic relatives to the human pathogen Kaposi sarcoma-associated herpesvirus (KSHV)—a gamma-2 herpesvirus and the etiologic agent of three malignancies associated with immunosuppression. In contrast to KSHV, RRV displays robust lytic-phase growth in culture, replicating to high titer, and therefore holds promise as an effective model for studying primate gammaherpesvirus lytic gene transcription as well as virion structure, assembly, and proteomics. More recently, investigators have devised complementary latent systems of RRV infection, thereby also enabling the characterization of the more restricted latent transcriptional program. Another benefit of working with RRV as a primate gammaherpesvirus model is that its efficient lytic growth makes genetic manipulation easier than that in its human counterpart. Exploiting this quality, laboratories have already begun to generate mutant RRV, setting the stage for future work investigating the function of individual viral genes. Finally, rhesus macaques support experimental infection with RRV, providing a natural in vivo model of infection, while similar nonhuman systems have remained resistant to prolonged KSHV infection. Recently, dual infection with RRV and a strain of simian immunodeficiency

virus (SIV) has led to a lymphoproliferative disorder (LPD) reminiscent of multicentric Castleman disease (MCD)—a clinical manifestation of KSHV infection in a subset of immunosuppressed patients. RRV, in short, shows a high degree of homology with KSHV yet is more amenable to experimental manipulation both in vitro and in vivo. Taken together, these qualities ensure its current position as one of the most relevant viral models of KSHV biology and infection.

1 Introduction

After the 1994 Moore and Chang discovery of Kaposi sarcoma-associated herpesvirus (KSHV) (Chang et al. 1994) and the subsequent series of investigations linking this virus to the human diseases Kaposi sarcoma (KS) (reviewed in Boshoff and Chang 2001), primary effusion lymphoma (PEL), and multicentric Castleman disease (MCD) (reviewed in Boshoff and Weiss 1998; Moore and Chang 2001), two separate groups isolated a homologous herpesvirus from rhesus macaques (*Macaca mulatta*). The first group was led by R. C. Desrosiers at the New England Primate Research Center (NEPRC) (Desrosiers et al. 1997) and the second by S. Wong at the Oregon Regional Primate Research Center (ORPRC) (Wong et al. 1999). Desrosiers' laboratory designated this new virus rhesus monkey rhadinovirus (RRV). Sequence analysis of the two separate isolates (Alexander et al. 2000; Searles et al. 1999) confirmed their classification as gamma-2-herpesviruses and demonstrated that the two were highly homologous to each other as well as to the human pathogen (Alexander et al. 2000; Searles et al. 1999).

The Desrosiers isolate, RRV 26-95H, originated from a colony of healthy monkeys at the NEPRC. The investigators originally noted that coculturing peripheral blood mononuclear cells (PBMC) from rhesus monkeys with primary rhesus fibroblasts (RhF) led to a cytopathic effect (CPE) in the latter cells. The investigators subsequently transfected primary RhF with RNase-treated virion DNA and propagated the virus on these same cells. Finally, they demonstrated that infection of the primary RhF with column-purified virus also led to similar CPE (Desrosiers et al. 1997).

In contrast to the discovery of the NEPRC isolate from healthy animals, the Wong group isolated RRV (isolate 17577 at the ORPRC) from SIV-infected macaques that had developed a lymphoproliferative disorder (LPD), characterized by splenomegaly, hepatomegaly, angiofollicular lymphadenopathy, and hypergammaglobulinemia, all of which are also clinical manifestations of MCD in humans (Bergquam et al. 1999). This group not only demonstrated that healthy animals in the same colony harbored a natural infection within circulating B lymphocytes (Bergquam et al. 1999) but also noted that

coinfection of healthy animals with SIV_{mac239} and RRV-17577 led to the development of LPD (Wong et al. 1999). Mansfield et al. experimentally infected macaques with RRV both alone and in the context of a different SIV strain, SIV_{mac251}. This coinfection resulted in no specific disease, although the investigators did find that the RRV 26-95H displayed a preferential tropism for CD20⁺ B lymphocytes (Mansfield et al. 1999). The reason for the different outcomes in these two sets of animal experiments remains unclear.

In the same year as the initial discovery of RRV, Rose et al. identified two additional herpesviruses in fixed tissue samples from macaques with simian retroperitoneal fibromatosis (RF) (Rose et al. 1997), a rare vascular tumor similar to KS (Giddens 1979). These investigators employed degenerative PCR primers to the highly conserved herpesvirus DNA polymerase genes to screen the RF tissue for a KSHV-related homologue. The RF tissue from both *Macaca nemestrina* and *Macaca mulatta* supported amplification of this region, and the investigators designated the predicted source viruses as RF-associated herpesvirus (RFHV)_{Mn} and RFHV_{Mm}, respectively (Rose et al. 1997). Subsequent sequence analysis of both isolates revealed an even closer homology to KSHV than RRV (Rose et al. 2003, 1997; Schultz et al. 2000; Strand et al. 2000). Rose and colleagues cloned over 4 kb of the RFHV_{Mn} and RFHV_{Mm} divergent locus B (DL-B) (Rose et al. 2003), a region of the genome that is 3' to the open reading frames (ORFs) encoding gB (ORF8) and the DNA polymerase (ORF9), both of which were sequenced previously by Schultz et al. (Schultz et al. 2000). Within the DL-B region these investigators identified viral homologues of cellular interleukin-6 (IL-6), dihydrofolate reductase (DHFR), and thymidylate synthase (TS), as well as the homologues of KSHV ORF10 and K3 (vMIR1) (Rose et al. 2003). Unfortunately, limited availability of well-preserved RF tissue and spontaneous disappearance of this tumor from the macaque colonies have prevented the isolation or growth of RFHV_{Mn} and RFHV_{Mm} in tissue culture (Rose et al. 2003). The remainder of this review, therefore, will focus on RRV.

2

RRV Genome

2.1

Genomic Organization

Analyses of the genomes of the two RRV isolates have demonstrated a high degree of similarity to each other, as well as to KSHV (Alexander et al. 2000). Nearly all of the genes in RRV have a homologue in KSHV (Fig. 1), although the monkey and the human viruses each have a small subset of



Fig. 1 Alignment of RRV and KSHV genomes. The ORFs are colored according to the code at the *bottom* of the figure. The *blunted and arrowhead ends* of each ORF signify 5' and 3' directionality of genes, respectively. *Numbers* designate each ORF and those preceded with *K* and *R* indicate those unique to RRV and KSHV, respectively. *Lines* connecting two ORFs designate spliced genes, and *TR* indicates terminal repeats. The *demarcated lines* specify the approximate length in kbp. (Adapted with permission from Alexander et al. 2000)

species-specific genes (see below). Sequence analysis of RRV17577 long unique region (LUR) and the characterization of the terminal repeats (TR) revealed that the genome was approximately 130,000 bp and had a G+C content of 52.2%. Sequence scanning predicted 79 ORFs, and 67 of these were similar to those of KSHV and herpesvirus saimiri (HVS). The same group also found eight genes encoded only by RRV and KSHV and four that are unique to RRV (Searles et al. 1999). Shortly thereafter, investigators at the NEPRC also sequenced the LUR of RRV isolate 26-95 (Alexander et al. 2000) and compared it to the published sequence for RRV17577 (Searles et al. 1999). The RRV26-95 LUR consists of 130,733 bp, and sequence analysis predicted an additional five ORFs for a total of 84. The overall sequence and genome architecture was nearly identical to the RRV17577 isolate and, again, highly similar to the KSHV LUR. Specifically, all of the ORFs of RRV26-95 have a homologue in KSHV, but the slightly shorter monkey virus genome lacks homologues of K3, K5 (vMIR2), K7 (viral inhibitor of apoptosis vIAP), and K12 (kaposin)—see below. In addition, RRV differs from KSHV in the number of ORFs encoding the macrophage inflammatory protein (MIP)-1 (RRV has 3 and KSHV 4) and viral interferon regulatory factors (vIRFs) (RRV has 1 and KSHV 8). Finally, the ORF encoding a viral homologue of cellular dihydrofolate reductase (DHFR) is located near the left end (5') of the genome in RRV26-95, as it is in HVS, whereas this ORF is displaced over 16 kbp rightward in KSHV and RFHV (Rose et al. 2003). The RRV26-95 DHFR ORF also retains a greater similarity to the HVS (74% similarity) than to the KSHV (55% similarity) DHFR homologue (Alexander et al. 2000).

The ORFs identified for RRV26-95 all have sequences similar to the ORFs of RRV17577, with a few minor exceptions. Sequences mapping to ORF67.5 of RRV26-95 are present in RRV17577 but were not originally designated as an ORF. Further, ORF R15 in RRV17577 is the sequence and positional homologue of R14 of RRV26-95 and K14 of KSHV (Alexander et al. 2000; Searles et al. 1999). Finally, meaningful direct comparisons between R15 and K15 await further characterization of the RRV protein products. Table 1 gives a detailed comparison among the two RRV isolates and KSHV. Together, these

Table 1 Comparison of the predicted proteins encoded by RRV isolates 26-95 and 17577 and KSHV (adapted with permission from Alexander, Denekamp et al. 2000)

ORF	RRV26-95	RRV17577		KSHV			Putative function	
	Size (aa)	Size (aa)	% Iden-tity	% Simi-larity	Size (aa)	% Iden-tity		% Simi-larity
R1	423	423	98.1	98.6	289	28	33	
2	188	188	99.9	99.5	210	45.5	54.4	DHFR
4	395	645	64.5	72.2	550	35.7	42.3	*CP
6	1132	1132	100	100	1133	63.3	71.3	ssDBP
7	686	686	99.6	99.6	695	52.2	60.8	TP
8	829	829	96.7	97.1	845	66.4	73.9	gB
9	1014	1014	100	100	1012	67.4	75.5	POL
10	416	416	100	100	418	35.3	44	dUTPase-related protein
11	409	409	100	100	407	33	42.8	dUTPase-related protein
R2	207	207	100	100	204	22.6	30.6	vIL-6
70	333	333	98.8	99.1	337	65.8	71.8	TS
R4	118	115	94.8	95.6	94	32.6	42.4	vMIP
16	187	187	100	100	175	46	58	vBcl-2
17	536	536	99.4	99.6	553	45.8	52.7	SCAF/PRO
18	257	299	100	100	257	58	68.1	Unknown
19	547	547	99.3	99.3	549	52.9	61.4	Packaging protein
20	350	350	99.4	99.4	320	46.9	53.9	Unknown
21	557	557	98.9	99.1	580	46.4	54.9	TK
22	726	704	74.2	79.4	730	41.2	50.8	gH
23	402	402	99.8	99.8	404	49	57.5	Egress protein
24	732	732	99.6	99.6	752	59.1	66.7	Unknown
25	1378	1378	99.6	99.6	1376	72.3	79.8	MCP
26	305	307	98.4	98.4	305	64.3	71.8	TRI-2
27	269	269	100	100	290	27.8	37.2	Unknown
28	91	91	100	100	102	26.5	30.1	gp150
29b	348	348	99.4	99.4	351	66.4	77.8	Packaging protein

Table 1 (continued)

ORF	RRV26-95	RRV17577		KSHV			Putative function	
	Size (aa)	Size (aa)	% Identity	% Similarity	Size (aa)	% Identity		% Similarity
30	76	76	98.7	98.7	77	38.2	51.3	Unknown
31	217	217	100	100	224	46.1	57.3	Unknown
32	464	464	100	100	454	42.9	51.2	Transport protein
33	336	336	100	100	312	42.7	52.8	MyrPBP
29a	327	327	100	100	312	61.2	66.7	Packaging protein
34	327	327	99.7	99.7	327	49.4	59.8	Unknown
35	149	149	100	100	151	37.2	49.3	Unknown
36	435	435	100	100	444	48.7	59.1	vPK
37	472	480	100	100	486	63.9	72.6	SOX
38	69	69	100	100	61	45	56.7	MyrP
39	378	378	100	100	399	59.5	73.3	gM
40	468	468	99.8	99.8	457	33	42.5	Helicase-primase
41	203	203	99.5	99.5	205	28.7	37.9	Helicase-primase
42	272	272	99.6	99.6	278	48	58.7	
43	576	576	100	100	605	62	70.1	PORT
44	790	790	100	100	788	66	74	Helicase-primase
45	353	352	99.4	99.4	407	37	43.6	vIRF-7BP
46	230	255	83.5	85.2	255	56.8	68.6	UDG
47	163	169	56.4	62	167	31.2	38.8	gL
48	389	389	100	100	402	29.6	39.1	Unknown
49	301	301	99.7	99.7	302	53.8	65.8	Unknown
50	514	514	99.7	100	631	44.9	54.7	RTA
R8								
R8.1								*8.1
52	139	139	100	100	131	47.7	60.8	Unknown
53	104	104	100	100	110	49	53.8	gN
54	290	290	100	100	318	43.1	51	dUTPase
55	210	210	100	100	227	55.2	62.8	PalmP

Table 1 (continued)

ORF	RRV26-95	RRV17577		KSHV			Putative function	
	Size (aa)	Size (aa)	% Iden-tity	% Simi-larity	Size (aa)	% Iden-tity		% Simi-larity
56	828	828	100	100	843	52.7	61.8	DNA replication protein
57	442	442	99.8	100	275	47.1	61.4	MTA
R9.1	415	415	98.3	98.8	449	28.7	60.6	vIRF
R9.2	415	415	99.5	99.5	449	23.2	37.2	vIRF
R9.3	351	351	100	100	449	24.3	31.9	vIRF
R9.4	361	253	99.6	99.6	449	25.5	34.2	vIRF
R9.5	385	385	100	100	449	29.5	38.4	vIRF
R9.6	390	290	100	100	449	22.9	33.4	vIRF
R9.7	355	355	99.4	99.4	449	25	34.6	vIRF
R9.8	364	364	100	100	449	26.8	33.6	vIRF
58	360	360	99.7	100	357	38.9	46.2	EpiL
59	394	394	100	100	396	51.8	60.2	PF
60	304	314	100	100	305	70.1	78.3	RNR-S
61	788	788	99	99.2	792	61.6	69.6	RNR-L
62	331	331	99.4	99.4	331	57.6	65.4	TRI-1
63	939	939	99.8	99.8	927	43.5	52.9	LTPBP
64	2548	2548	99.6	99.6	2635	41.3	51.2	LTP
65	169	169	100	100	170	45.5	55.8	SCIP
66	448	448	99.8	99.8	429	47.3	52.7	Egress protein
67	222	224	87.8	87.8	271	67.1	71.2	Unknown
67.5	86	86	98.8	98.8	80	62.5	71.2	Unknown
68	457	457	100	100	545	49.1	58.3	Unknown
69	297	297	100	100	225	65.6	73.1	Unknown
R13	174	174	99.4	99.4	188	33.1	40.1	FLIP
72	254	254	99.6	99.6	257	39.4	50.2	vCyclin D
73	448	447	99.8	97.8	1162	18.3	34.9	LANA
R14	253	253	100	100	348	32.8	37.6	vOx2
74	342	342	100	100	342	42	54.7	vGPCR
75	1298	1298	99.8	99.9	1296	44.9	53.2	vFGARAT
R15					490			LAMP

* Protein named accordingly: R, for RRV; K, for KSHV

data demonstrate that the independent RRV isolates are the same virus species and that both are highly related to KSHV in both structural organization of the genome and overall sequence (Alexander et al. 2000; Searles et al. 1999).

2.2

Genomic Structure—Conservation with KSHV

The 130.7-kbp LUR of RRV with its 84 predicted ORFs, is approximately colinear with KSHV (Fig. 1). As we mentioned above, four genes are unique to KSHV and absent in the RRV genome. These include K3 and K5 [bovine herpesvirus immediate early protein 4 (IEP4) homologues involved in immune modulation], K7 (vIAP), and K12 (kaposin, a transforming gene). The absence of a homologue to K12 is particularly noteworthy, as this is the most abundantly transcribed region of the genome in KSHV latent infection and, thus, may be a potentially important clue to understanding the differences in the biology and/or pathogenesis of the two viruses. Interestingly, adjacent to the K12 region of the KSHV genome are two G+C-rich direct repeating units, DR-1 and DR-2. Although RRV lacks the K12 homologue, it has retained the G+C-rich repeating elements in positions that are similar to those of DR-1 and DR-2 within the KSHV genome, suggesting that the monkey virus may have lost this gene during evolution from a common herpesvirus ancestor (Alexander et al. 2000). The eight genes specific to RRV and KSHV are the homologues to interleukin-6 (vIL-6), macrophage inflammatory proteins (vMIPs), interferon regulatory factors (vIRFs), Fas-associating protein with death domain-like interleukin-1b converting enzyme (FLICE) inhibitory protein (vFLIP), vOx-2, and vIL-8 receptor (Alexander et al. 2000). In addition, although RRV's vIRFs show little similarity to those of KSHV, the RRV vIRFs have a high degree of similarity to each other, suggesting an amplification event in which the four ancestral vIRFs duplicated, yielding the eight now present in the genome (Alexander et al. 2000; Searles et al. 1999). The vIRFs encoded by R6, R7, R8, and R9 are most similar to those encoded by R10, R11, R12, and R13, respectively. R6, R7, R8, R10, and R11 are similar to K9, but only R10 has higher than 30% similarity to K9. Thus, Searles et al. suggest that a possible duplication event occurred, such that the four vIRFs of KSHV were duplicated en masse, creating the eight now present in the RRV genome (Searles et al. 1999).

RRV and KSHV also share a high degree of homology in nucleotide sequence as well as the G+C distribution at the origin of lytic replication (OriLyt) (Pari et al., 2001), although the location and number of OriLyts in RRV differ from those in KSHV (AuCoin et al. 2002). The RRV OriLyt maps between ORF69 and ORF71 and has an A+T region that is followed by a short, downstream G+C-rich sequence (Pari et al. 2001). KSHV has a left- and a right-end

OriLyt, with the latter showing the most similarity to the OriLyts of RRV (AuCoin et al. 2002).

3

RRV Transcriptional Program and Characterization of Individual Proteins

Recent reports on the transcription program of RRV also strengthen the argument for using RRV as a model system for the study of KSHV. After the cloning of full-length cDNAs for replication and transcription activator (RTA), investigators showed that RTA is a strong transcriptional transactivator of early RRV promoters, including those of R8, ORF57, and gB. Because KSHV RTA activates the promoters of the KSHV homologues of these same three genes, this work helped demonstrate the conservation of RTA function between RRV and KSHV (DeWire et al. 2002). In further support of using RRV to study KSHV transcription, Dittmer and colleagues recently described the overall transcription program of RRV after *de novo* infection (Dittmer et al., 2005). Using real-time reverse transcriptase (RT)-based polymerase chain reaction (PCR) techniques to profile the whole genome of RRV after infection of RhFs, they found that the lytic transcription program of RRV closely resembles that of KSHV after the induction of lytic reactivation (Dittmer et al. 2005). However, in contrast to KSHV culture systems that are marked by inefficient reactivation from a latent infection, *de novo* infection of RhFs with RRV results in highly efficient spontaneous lytic replication. Thus, RRV affords a powerful advantage for studying primate gammaherpesvirus lytic replication.

Although they share limited sequence homology, two transmembrane signaling molecules, R1 and K1, are unique to RRV and KSHV, respectively, and both contribute to cell growth and transformation (reviewed in Damania et al. 1999a), although the mechanism(s) by which this occurs remains poorly understood. Both K1 and R1 oligomerize through disulfide bonding (Damania et al. 1999b; Lee et al. 1998), transmit extracellular signals into the cytoplasm through ITAM motifs, and also share corresponding positions, located at the leftmost end of their respective genomes. Recently, L. Wang and colleagues demonstrated that K1 expression results in vascular endothelial growth factor (VEGF) promoter activation, leading to the expression and secretion of this growth factor, and also induces matrix metalloproteinase-9 (MMP-9) expression in endothelial cells. These results suggest that K1 may play a role in KSHV pathogenesis by inducing the expression of these proteins involved in both angiogenesis and invasion (Wang et al. 2004). Finally, using a recently described latent system of infection (see below), investigators have found that the RRV latency-associated nuclear antigen (LANA), significantly shorter than

its KSHV counterpart, is also expressed during the lytic phase of infection and that RRV LANA inhibits lytic replication through the repression of RTA transcription (DeWire and Damania 2005). KSHV LANA, more intensely studied than its RRV counterpart, has multiple functions, including a role in tethering the viral genome to host cell chromosomes during latency (Cotter and Robertson 1999; Cotter et al. 2001; Garber et al. 2001; Grundhoff and Ganem 2003; Hu et al. 2002). Additionally, KSHV LANA regulates viral and cellular promoters, including its own (Jeong et al. 2001), acting as either an activator or a repressor of transcription (Schwam et al. 2000). The full extent of the parallels between the LANA homologues of KSHV and RRV awaits further investigation.

4

In Vivo and In Vitro Infection with RRV

RRV serves as a useful model system for the study of KSHV both in vivo and in vitro. In contrast, KSHV appears unable to persistently infect either mice or rhesus macaques—a major limitation in the study of the human virus (Dittmer et al. 1999; Renne et al. 2004). As described above, the natural host of RRV, rhesus macaques, can serve as a primate model system to study RRV biology and pathogenesis following de novo infection. Even for in vitro work RRV offers some advantages over, and can complement direct work with KSHV. Specifically, one of the major difficulties facing KSHV investigators interested in studying the viral transcription program is that only a small percentage (25%–30% or less) (Chan et al. 1998) of latently infected PEL cells (a B cell line) in culture respond to chemical induction and support lytic reactivation. Furthermore, of these only a fraction (approximately 25%) complete the lytic cycle and actually produce virus (Renne et al. 1996). More recently, transduction of the lytic “switch” gene, ORF50 (encoding RTA), into these cells led to higher proportions of the culture initiating the lytic cycle (Bechtel et al. 2003). Nevertheless, with KSHV culture systems, the background of simultaneous latent gene expression and frequently incomplete lytic reactivation complicates the study of the lytic gene transcription program. These in vitro KSHV systems are likewise not ideal for latent studies, because the converse complication exists with low but significant levels of spontaneous lytic reactivation remaining within 2%–5% of cells in the absence of any exogenous stimulation (Miller et al. 1997; Zhong et al. 1996). Although investigators have had some success at KSHV de novo infection of endothelial cells, the inability to reactivate efficiently the resultant latent infection and obtain high titers or to passage the virus over extended periods after de novo infection remains a major impediment to the

development of a tractable genetic system similar to those that exist for other herpesviruses such as herpes simplex virus-1 (HSV-1), for example (Dezube et al. 2002; Lagunoff et al. 2002; Renne et al. 1998; Sakurada et al. 2001).

Infection of RhFs with RRV, in contrast, yields high titers of virus (e.g., 10^6 PFU/ml) in culture media within 6 days after infection (O'Connor et al. 2003). Further, the kinetics of RRV gene expression after infection of RhFs resembles closely that of KSHV lytic reactivation (DeWire et al. 2002). Two separate RhF cell lines are available, and both are permissive to RRV infection. In both cases, the cells are immortalized with telomerase, facilitating serial passaging (DeWire et al. 2002; Kirchoff et al. 2002). Unlike the KSHV in vitro system, infection of the RhFs with RRV is in the context of de novo infection (Alexander et al. 2000). The RRV in vitro system also allows researchers to perform traditional plaque assays (DeWire, 2003), a technique, that has eluded current KSHV in vitro systems.

Recently, DeWire and Damania described a latent system of RRV infection (DeWire and Damania 2005). After infection, both BJAB and 293-HEK cells harbor latent RRV and the virus can be reactivated with the addition of TPA. Additionally, transient transfection of RRV ORF50, which encodes the lytic switch protein RTA, into the BJAB cell line results in approximately four-fold reactivation of the viral lytic life cycle (DeWire and Damania 2005). Both the lytic and latent in vitro systems of RRV infection provide useful tools for the study of primate gammaherpesviruses in culture.

Additionally, naive rhesus monkeys are susceptible to experimental infection with RRV (Mansfield et al. 1999; Wong et al. 1999), and the manifestations of infection approximate many of the clinical diseases of KSHV infection in humans. Infection of RRV-naive, SIV-positive macaques with RRV-17577, for example, led to the development of a hyperplastic B lymphocytic LPD, resembling MCD observed in patients dually infected with KSHV and HIV (Wong et al. 1999). In addition, experimental infection of immunocompetent, SIV-negative animals coinfecting with RRV 26-95 resulted in clinical lymphadenopathy characterized initially by paracortical hyperplasia and vascular hypertrophy/hyperplasia that was subsequently replaced by follicular hyperplasia (Mansfield et al. 1999). Similarly, HIV-negative MCD patients who harbor KSHV also present with B cell proliferation and angioimmunoblastic lymphadenopathy. In this same study, three of the four animals developed arteriopathy that resembled the vascular endothelial lesions observed in KS patients, and therefore the authors suggest a possible role for RRV in development of this lesion (Mansfield et al. 1999). Because small-animal models capable of long-term propagation of KSHV are still only in initial stages of development (Parsons et al. 2006), the in vivo system of RRV is an attractive model system for the study of these two closely related viruses.

5 Structural Conservation Between RRV and KSHV Capsids

KSHV lytic replication leads to the production of three (denoted A, B, and C) biochemically and structurally distinct capsid species (Nealon et al. 2001), similar to those in other herpesviruses (reviewed in Homa and Brown, 1997). A capsids are empty icosahedral structures (Fig. 2A), B capsids contain an inner ringlike structure (Fig. 2B), presumably the viral scaffolding (SCAF), and C capsids contain the viral DNA (Fig. 2C). The C capsids most likely mature to virions, after gaining tegument and envelope proteins. Because the KSHV culture system does not permit efficient, productive lytic reactivation, the yield of these capsid species is also low. Among the KSHV capsids that lytic reactivation does produce, the C capsids are most poorly represented, whereas A- and B-type capsids predominate (Fig. 3) (Nealon et al. 2001). For these reasons, studying the capsid assembly and maturation of KSHV capsids into virions is particularly challenging, and, as we describe below, RRV culture systems offer an attractive alternative.

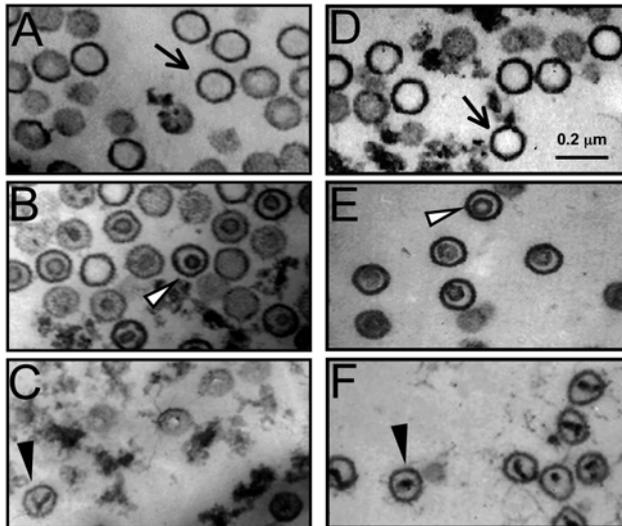


Fig. 2A–F TEMs of KSHV and RRV capsids. A–C KSHV capsids. D–F RRV capsids. A, D A capsids (*arrows*) are empty icosahedral structures. B, E B capsids (*white arrowhead*) have an inner ringlike structure, likely composed of SCAF. C, F C capsids (*black arrowheads*) contain a dense core consistent with encapsidated viral DNA. Bar indicates 0.2 μm . (Adapted with permission from Nealon et al. 2001 and O'Connor et al. 2003)

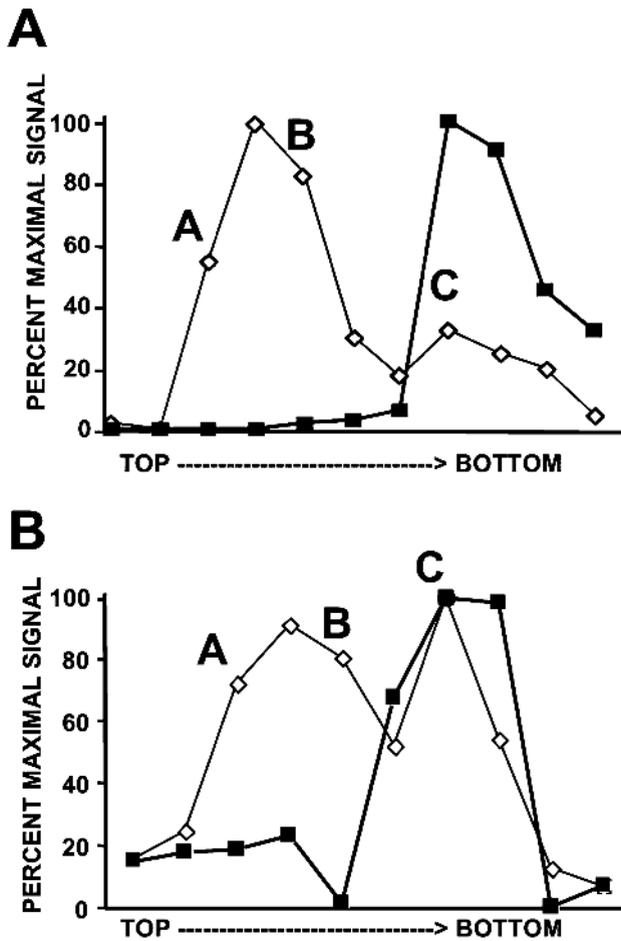


Fig. 3A, B Encapsitated viral DNA cosediments with C capsids—the predominant species in RRV. The MCP profiles (*open diamonds*) across fractions from sucrose gradients reveal distinct sedimentation velocities of the three different KSHV and RRV capsid species (identified by TEM, see Fig. 2; the letters A, B, and C designate the fractions containing the each of the isolated populations, respectively). The viral DNA profile (*filled squares*) from these same fractions confirms the location of C capsids. **A** KSHV A- and B-type capsids predominate (measured by intensity of Coomassie-stained MCP), whereas the C capsids are in low abundance. **B** In contrast, RRV C capsids are the most abundant capsid type, corresponding with the peak of viral DNA. (Adapted with permission from Nealon et al. 2001 and O'Connor et al. 2003)

De novo RRV infection of RhFs is, in contrast to KSHV reactivation, highly efficient *in vitro* (see above). Like KSHV, RRV lytic replication results in the synthesis of the same three capsid types, the A, B, and C capsids (Fig. 2D–F) (O'Connor et al. 2003). The RRV capsids are comprised of proteins similar to those of the KSHV capsids. All three capsid species in the two viruses contain the major capsid protein (MCP/ORF25), the triplex proteins TRI-1 (ORF62) and TRI-2 (ORF26), and the small capsomer interacting protein (SCIP/ORF65). Additionally, the B capsids contain the scaffolding protein (SCAF), and the C capsids, instead, contain the viral DNA (Fig. 2E and F). Structural analyses of these particles revealed that they were highly similar to those of KSHV, in both transmission electron (Fig. 2) (O'Connor et al., 2003) and cryoelectron (cryoEM) microscopic and three-dimensional (3D) reconstruction studies (Fig. 4) (Yu et al. 2003). Furthermore, the elevated levels of RRV capsids have led to high-resolution reconstructions for the three RRV capsid types, representing the first reconstruction of the A, B, and C capsids for gammaherpesviruses (Yu et al. 2003). In contrast to patterns of capsid production during KSHV replication, RRV replication results in high

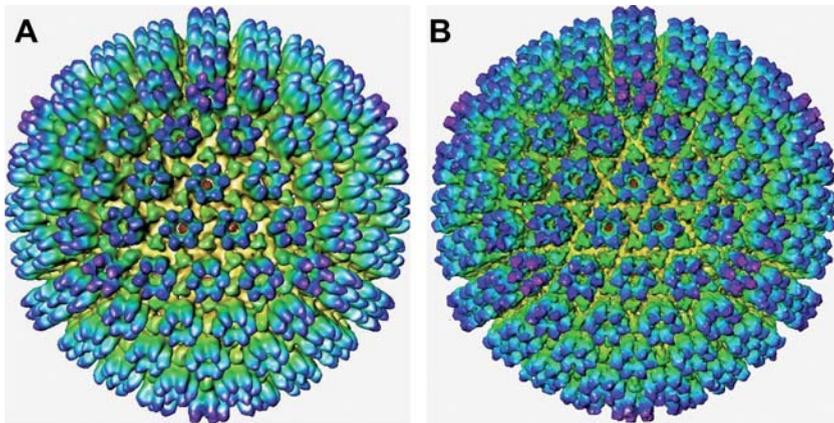


Fig. 4A, B Three-dimensional reconstructions of the RRV and KSHV capsids. The KSHV (A) and RRV (B) capsids are reconstructed to 15-Å and 24-Å resolution, respectively, and viewed along the icosahedral threefold axis. The maps are color coded according to the particle radius, such that the upper domains of the pentons and hexons are in *blue* (between radii of 570 and 640 Å), the connecting triplexes are in *green* (between radii of 510 and 560 Å), and the shell is in *yellow* (between radii of 460 and 510 Å). The higher resolution of the RRV compared to the KSHV capsid results from the integration of greater numbers of more highly purified RRV particles and allows better definition of the individual protein components. (Figure provided by Z. Hong Zhou and Xuekui Yu and modified, with permission, from Wu et al. 2000 and Yu et al. 2003)

proportions of the genome-filled C capsids (Fig. 3) (O'Connor 2005). The most abundant capsid species produced after KSHV lytic reactivation is the A capsid (Fig. 3A) (Nealon et al. 2001). In contrast, the genome-filled C-type capsid is the most prevalent capsid type produced after *de novo* infection of RhF with RRV (Fig. 3B) (O'Connor 2005). This difference in the production of the genome-containing C capsids provides the first potential explanation for the differences in replication efficiency (production of mature virions) between the two viruses.

6

Structural Conservation Between RRV and KSHV Virions

Accurately determining the protein composition of mature herpesvirus particles is essential to understanding not only their assembly but also early events following infection. Further, the ways in which KSHV and RRV rapidly initiate changes to the host cell are most likely distinct from other herpesviruses because they also differ in their modes of transmission, host cell tropism, balance of latent and lytic replication, and pathogenesis. Recently, two groups working on KSHV have reported their findings on virion-associated proteins with mass spectrometric (MS) techniques (Bechtel et al. 2005; Zhu et al. 2005). Bechtel et al. induced KSHV lytic replication of five liters of latently infected BCBL-1 cells, treated gradient-purified virus with trypsin, and separated the viral proteins by SDS-PAGE. By excising visibly stained gel slices, the investigators identified a total of 18 proteins, 10 of which are encoded by the virus (Table 2) (Bechtel et al. 2005). In a similar study by Zhu et al., KSHV lytic replication was reactivated in BCBL-1 cells, and the virus was subsequently purified by both step- and continuous gradient centrifugation. After separation of the viral proteins by SDS-PAGE, the investigators excised visible bands for MS analysis. In this study, the investigators reported a total of 26 proteins associated with the virus (24 encoded by the virus and 2 by the cell) (Table 2) (Zhu et al. 2005).

Taking advantage of the high yield of virus from RRV infection of RhF in culture, our laboratory has recently optimized the purification methods used to isolate both virions and capsids. In contrast to KSHV culture systems, much smaller (<1 or 2 liters) preparations of RRV-infected media are able to provide sufficient material to allow similar MS analyses (O'Connor and Kedes 2006). In our work, we treated virions with one of two proteases before gradient centrifugation, eliminating any contaminating cellular or viral proteins stuck nonspecifically to the outside of the particles and thus ensuring the purity of the particles. We used both velocity sedimentation and density

Table 2 Virion proteins of RRV and KSHV

Name	Abbrev.	RRV	KSHV	EBV	MHV-68	HSV-1	VZV	HCMV	HHV6/7	RRV	KSHV ¹	KSHV ²	MHV-68 ³	EBV ⁴	HCMV ⁵
<i>Capsid</i>															
Major capsid protein	MCP	ORF25	ORF25	BcLF1	ORF25	UL19	40	UL86	U57	+	+	+	+	+	+
Triplex-1	TRI-1	ORF62	ORF62	BORF1	ORF62	UL38	20	UL46	U29	+	+	+	+	+	+
Triplex-2	TRI-2	ORF26	ORF26	BDLF1	ORF26	UL18	41	UL85	U56	+	+	+	+	+	+
Small capsomer interacting protein	SCIP	ORF65	ORF65	BFRF3	ORF65	UL35	23	UL48.5	U32	+	-	+	+	+	+
Portal protein	PORT	ORF43	ORF43	BBRF1	ORF43	UL6	54	UL104	U76	+	-	-	-	+	-
Packaging protein	-	ORF19	ORF19	BVRF1	ORF19	UL25	34	UL77	U50	+	-	-	-	+	-
Scaffolding protein	SCAF	ORF17.5	ORF17.5	BdRF2	ORF17.5	UL26.5	33.5	UL80.5	U53.5	+	-	+	-	+	-
Protease	PRO	ORF17	ORF17	BVRF2	ORF17	UL26	33	UL80	U53	+	-	-	-	-	+
<i>Tegument</i>															
DNA binding protein	ssDBP	ORF6	ORF6	BALF2	ORF6	UL29	29	UL57	U41	-	-	+	-	+	+
Transport protein	TP	ORF7	ORF7	BALF3	ORF7	UL28	30	UL56	U40	-	-	+	-	-	-
DNA polymerase	POL	ORF9	ORF9	BALF5	ORF9	UL30	28	UL54	U38	-	-	-	-	-	+

Table 2 (continued)

Name	Abbrev.	RRV	KSHV	EBV	MHV-68	HSV-1	VZV	HCMV	HHV6/7	RRV	KSHV ¹	KSHV ²	MHV-68 ³	EBV ⁴	HCMV ⁵
dUTPase-related protein	-	ORF11	ORF11	LF2	ORF11	γ	γ	γ	γ	-	-	+	-	γ	γ
Unknown	-	ORF20	ORF20	BXRF1	ORF20	UL24	35	UL76	U49	-	-	-	+	-	-
Thymidine kinase	TK	ORF21	ORF21	BXLF1	ORF21	UL23	36	n/a	n/a	+	+	+	-	+	n/a
Egress protein	-	ORF23	ORF23	BTRF1	ORF23	UL21	38	UL88	U59	+	-	-	-	low	+
Unknown	-	ORF24	ORF24	BcRF1	ORF24	n/a	n/a	UL87	U58	-	+	-	+	-	-
Unknown	-	ORF27	ORF27	BDLF2	ORF27	γ	γ	γ	γ	+	-	+	-	+	γ
Packaging protein	-	ORF29	ORF29	BGRF1/BDRF1	ORF29	UL15	42/45	UL89	U66	-	-	-	+	-	+
Transport protein	-	ORF32	ORF32	BGLF1	ORF32	UL17	43	UL93	U64	+	-	-	-	+	+
Myristoylated protein binding protein	MyrPBP	ORF33	ORF33	BGLF2	ORF33	UL16	44	UL94	U65	+	+	+	-	+	+
Unknown	-	ORF35	ORF35	BGLF3.5	ORF35	UL14	46	UL95	U67	+	-	-	-	low	-
Virion protein kinase	vPK	ORF36	ORF36	BGLF4	ORF36	UL13	47	UL97	U69	+	-	-	-	+	+
Shutoff exonuclease	SOX	ORF37	ORF37	BGLF5	ORF37	UL12	48	UL98	U70	-	-	-	-	-	-

Table 2 (continued)

Name	Abbrev.	RRV	KSHV	EBV	MHV-68	HSV-1	VZV	HCMV	HHV6/7	RRV	KSHV ¹	KSHV ²	MHV-68 ³	EBV ⁴	HCMV ⁵
Myristoylated MyrP protein	-	ORF38	ORF38	BBLF1	ORF38	UL11	49	UL99	U71	+	-	-	-	+	+
vIRF-7 binding protein	-	ORF45	ORF45	BKRF4	ORF45	UL7	53	UL103	U75	+	-	-	low	+	+
Unknown	-	ORF48	ORF48	BRRF2	ORF48	γ	γ	γ	γ	+	-	-	+	+	γ
Unknown	-	ORF49	ORF49	BRRF1	ORF49	γ	γ	γ	γ	+	-	-	-	-	γ
Unknown	-	ORF52	ORF52	BLRF2	ORF52	γ	γ	γ	γ	+	-	+	+	+	γ
dUTPase	-	ORF54	ORF54	BLLF3	ORF54	UL50	8	UL72	U45	+	-	-	low	+	+
Palmitoylated protein	-	ORF55	ORF55	BSRF1	ORF55	UL51	7	UL71	U44	+	-	-	-	+	+
mRNA transcript	-	ORF57	ORF57	BSLF1/MLF1	ORF57	UL54	4	UL69	U42	-	-	-	-	-	+
accumulation function	-	ORF59	ORF59	BMRF1	ORF59	UL42	16	UL44	U27	-	-	-	-	+	+
Processivity factor	-	ORF60	ORF60	BaRF1	ORF60	UL40	18	n/a	n/a	-	-	-	low	n/a	n/a
Small ribonucleotide reductase	-	ORF61	ORF61	BORF2	ORF61	UL39	19	UL45	U28	-	-	-	-	+	+

Table 2 (continued)

Name	Abbrev.	RRV	KSHV	EBV	MHV-68	HSV-1	VZV	HCMV	HHV6/7	RRV	KSHV ¹	KSHV ²	MHV-68 ³	EBV ⁴	HCMV ⁵
Large tegument protein binding protein	LTPBP	ORF63	ORF63	BOLF1	ORF63	UL37	21	UL47	U30	+	+	+	-	+	+
Large tegument protein	LTP	ORF64	ORF64	BPLF1	ORF64	UL36	22	UL48	U31	+	-	+	-	-	+
Egress protein	-	ORF66	ORF66	BFRF2	ORF66	UL34	24	UL50	U34	-	-	-	-	low	+
Unknown	-	ORF67	ORF67	BFRF1A	ORF67	UL33	25	UL51	U35	-	-	-	-	-	+
Unknown	-	ORF68	ORF68	BFLF1	ORF68	UL32	26	UL52	U36	-	-	+	-	-	-
FGARAT homolog	vFGA-RAT	ORF75	ORF75	BNRF1	ORF75	γ	γ	γ	γ	+	+	+	+	+	γ
Unknown	-	γ1	γ1	BLLF1	γ1	γ1	γ1	γ1	γ1	γ1	γ1	γ1	γ1	+	γ1
Unknown	-	γ1	γ1	BZLF2	γ1	γ1	γ1	γ1	γ1	γ1	γ1	γ1	γ1	+	γ1
Unknown	-	γ1	γ1	BILF2	γ1	γ1	γ1	γ1	γ1	γ1	γ1	γ1	γ1	+	γ1
<i>Envelope</i>															
Complement binding protein	*CP	ORF4	ORF4	γ2	ORF4	γ2	γ2	γ2	γ2	+	-	-	-	γ2	γ2
Glycoprotein B	gB	ORF8	ORF8	BALF4	ORF8	UL27	31	UL55	U39	+	+	+	+	+	+

Table 2 (continued)

Name	Abbrev.	RRV	KSHV	EBV	MHV-68	HSV-1	VZV	HCMV	HHV6/7	RRV	KSHV ¹	KSHV ²	MHV-68 ³	EBV ⁴	HCMV ⁵
Glycoprotein *8.1		ORF R8.1	ORF K8.1 ‡		‡	‡	‡	‡	‡	+	-	+	‡	‡	‡
Glyco-protein H	gH	ORF22	ORF22	BXLF2	ORF22	UL22	37	UL75	U48	+	+	+	+	+	+
Glyco-protein 150	gp150	ORF28	ORF28	BDLF3	ORF28	Y	Y	Y	Y	+	-	+	+	+	Y
Glyco-protein M	gM	ORF39	ORF39	BBRF3	ORF39	UL10	50	UL100	U72	+	-	+	-	+	+
Glyco-protein L	gL	ORF47	ORF47	BKRF2	ORF47	UL1	69	UL115	U82	+	-	+	-	+	+
Glyco-protein N	gN	ORF53	ORF53	BLRF1	ORF53	UL49A	9A	UL73	U46	+	-	+	-	+	+
Epithelial ligand	EpiL	ORF58	ORF58	BMRF2	ORF58	Y	Y	Y	Y	+	-	-	-	+	Y

Data from ¹ Bechtel, J.T., et al. (2005); ² Zhu, F.X., et al. (2005); ³ Bortz, E., et al (2003); ⁴ Johannsen, E., et al. (2004); ⁵ Varnum, S.M., et al. (2004)

Y gammaherpesvirus-specific protein

γ1 gamma-1 herpesvirus-specific protein, and not detected in γ2

γ2 gamma-2 herpesvirus-specific protein, and not detected in γ1

* Protein named accordingly: R, for RRV; K, for KSHV

‡ Protein only encoded by RRV and KSHV

n/a Protein not encoded by the respective virus

“low” Signifies those proteins the investigators found in low abundance and did not include in their final list of EBV-associated proteins

centrifugation and analyzed the particles with two different MS techniques: multidimensional protein identification technology (MudPIT) and tandem MS of excised gel slices. We identified 33 virally encoded proteins associated within RRV. These included seven capsid proteins, 17 putative tegument proteins, and nine envelope proteins. Five of the 33 proteins are gammaherpesvirus specific, and three of these have no known function. Additionally, we identified three proteins not previously associated with a herpesvirus, and seven are new findings for a gamma-2-herpesvirus (O'Connor and Kedes 2006). In addition to the five capsid proteins we previously demonstrated as RRV capsid components (O'Connor et al. 2003), we identified the portal protein (PORT/ORF43) and the packaging protein (ORF19) (O'Connor and Kedes 2006). The 17 proteins we assigned to the tegument are tentative assignments, as their localization is based on homology with other herpesviruses, and thus require direct experimental evidence. Five of the 17 putative tegument proteins are gammaherpesvirus specific, and three of these five have no known function. Three of the nine envelope proteins are gammaherpesvirus specific, including the KSHV complement control protein (KCP) homologue encoded by ORF4. Finally, after detergent treatment of purified virions, a subset (six) proteins remain associated with the capsid, suggesting that these proteins interact more directly with and/or with higher affinity for the underlying capsid. We hypothesize that these last proteins may play a role in assembly or transport of viral or subviral particles during entry or egress (O'Connor and Kedes 2006).

Although the multiple techniques and rigorous purification methods are a potential explanation for the increased number of proteins identified in this RRV study, it is also likely that the greater abundance of purified virions played a major role. Because RRV infection of RhFs result in a greater number of particles produced, the loss we potentially incur throughout our purification protocol is not as significant as with KSHV, where the starting material is not as great. In the case of KSHV, this loss is detrimental as this, in turn, reduces the amount of protein for staining after SDS-PAGE separation. For the more abundant virion proteins, such as MCP (present in 960 copies/capsid), this is of little to no consequence, but for proteins that are present in the virions in low abundance, such as the portal protein (PORT/ORF43) present in only 12 copies/capsid, this can be a significant factor in protein detection. It is possible, therefore, that because of the low yield of KSHV virions, coupled with sample loss throughout the protocol, some of the less abundant virion-associated proteins were not detected in the KSHV studies but, in contrast, were found in the RRV investigation.

Thus the RRV system allows the detailed study of the structure and proteomic composition of gamma-2-herpesviruses. Defining the molecular composition of this primate gammaherpesvirus has begun to lay the foundation for the next stage of this research—to explore true contributions of cellular components of the mature virion and to define the functions of each of these virion proteins, examining their potential roles in initial infection, entry, assembly, and egress.

7

The Future of RRV as a Model for the Study of KSHV

With its high level of sequence and genome conservation with KSHV, efficient lytic growth in cell culture, and ability to infect naive rhesus monkeys, RRV clearly remains a highly attractive model system for studying primate gammaherpesvirus biology. Further, the RRV lytic system allows for genetic manipulation of the virus, including the production of recombinant and mutant viruses. Recently, DeWire et al. developed an RRV-GFP construct, in which the gene encoding GFP was inserted into the genome between two ORFs. This RRV-GFP and wild-type RRV replicate to similar titers and with similar kinetics (DeWire et al. 2003), providing clear support for the feasibility and usefulness of these methods in the study of the virus. It will also undoubtedly be equally amenable to siRNA and bacterial artificial chromosome (BAC) techniques of genetic manipulation that together will aid in characterizing the overall biology as well as the functions of individual proteins encoded by the virus. We anticipate that future work with RRV will continue to give insights into not only the mechanisms underlying its own replication, pathogenesis, structure and assembly, and persistence but also those of its human homologue, KSHV.

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The Rta/Orf50 Transactivator Proteins of the Gamma-Herpesviridae

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1	Introduction	72
2	Immediate-Early Genes	72
3	Lytic Reactivation	74
3.1	Lymphocryptovirus—EBV	75
4	Rhadinoviruses—KSHV, HVS, RRV, and MHV-68	76
4.1	Experimental Considerations	76
4.2	Chemically Induced Viral Reactivation of Gammaherpesviruses	77
4.3	Viral Induction of Lytic Reactivation of Rhadinoviruses	78
5	Rta/Orf50 Transcription	78
5.1	Regulation of the KSHV Rta/Orf50 Promoter	79
6	Rta/Orf50 Protein	81
6.1	KSHV Rta/Orf50	81
6.2	MHV-68 Rta/Orf50	82
6.3	HVS Rta/Orf50	83
7	Rta/Orf50 Function	84
7.1	Viral Promoters Transactivated by KSHV Rta/Orf50	84
7.2	Viral Promoters Transactivated by MHV-68 Rta/Orf50	84
7.3	Viral Promoters Transactivated by RRV Orf50	84
7.4	Viral Promoters Transactivated by HVS Orf50	85
8	Mechanisms of Rta/Orf50 Transactivation	86
8.1	KSHV Orf50-Responsive Elements and Direct DNA Binding	86
8.2	Interaction of Rta/Orf50 with RBP-J- κ	87
8.3	Interaction of Rta/Orf50 with Other Cellular Transcription Factors	88
9	Repression of Rta/Orf50 Transactivation	89
	References	91

Abstract The replication and transcription activator protein, Rta, is encoded by *Orf50* in Kaposi's sarcoma-associated herpesvirus (KSHV) and other known gammaherpesviruses including Epstein-Barr virus (EBV), rhesus rhadinovirus (RRV), herpesvirus saimiri (HVS), and murine herpesvirus 68 (MHV-68). Each Rta/Orf50 homologue of each gammaherpesvirus plays a pivotal role in the initiation of viral lytic gene expression and lytic reactivation from latency. Here we discuss the Rta/Orf50 of KSHV in comparison to the Rta/Orf50s of other gammaherpesviruses in an effort to identify structural motifs, mechanisms of action, and modulating host factors.

1

Introduction

As all members of the *Herpesviridae*, the gammaherpesviruses can establish either a latent or lytic life cycle within host cells. During latency, only a few viral genes are transcribed and the virus exists as a nonintegrated circular episome within the nucleus of the infected cell (Fakhari and Dittmer 2002; Jenner et al. 2001; Paulose-Murphy et al. 2001; Sarid et al. 1998; Zhong et al. 1996). B cell latency can be disrupted by host cell signaling, such as B cell receptor cross-linking, which leads to the sequential expression of several subsets of lytic genes: immediate-early genes (IE) that encode viral transcriptional regulators; delayed-early genes (DE) that encode proteins involved in viral DNA replication, and late genes (L) that encode viral structural proteins. Herpesvirus lytic gene expression follows this temporal and sequential cascade, ultimately resulting in the production of progeny virions and destruction of and egress from the infected host cell. The Rta/Orf50 switch protein is essential to initiate lytic reactivation of all gammaherpesviruses: Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), rhesus rhadinovirus (RRV), herpesvirus saimiri (HVS), and murine herpesvirus 68 (MHV-68).

2

Immediate-Early Genes

Immediate-early genes define mRNAs that are transcribed in the presence of protein synthesis inhibitors, such as cycloheximide. This applies to herpesvirus genes after de novo infection of permissive cells (Roizman 1996) but also to cellular genes after serum stimulation. Lau and Nathans identified cellular immediate-early genes (*jun/fos*) because they constituted the first wave of mRNAs after serum stimulation of mouse fibroblasts (Lau and Nathans 1985, 1987). *jun/fos* mRNA levels were induced within 10 min after addition of

serum and declined shortly thereafter. By comparison, the induction of *c-myc*, a cellular early mRNA, was delayed. *c-Myc* mRNA peaked at 20–45 min, after the wave of immediate-early mRNAs subsided, and stayed induced for longer periods of time. The definition of early genes for herpesviruses is more strict: Early gene transcription is dependent on immediate-early transactivators independent of the time frame. Herpesvirus immediate-early transactivators are necessary and sufficient to initiate viral replication (McKnight et al. 1987; Triezenberg et al. 1988).

Rta/Orf50 is an immediate-early protein of rhadinoviruses. It is necessary and sufficient to drive lytic replication for KSHV, HVS, RRV, and MHV-68. Ectopic expression of Rta/Orf50 will reactivate virus from latency (sufficient); deletion of Rta/Orf50 or inhibition by a dominant-negative mutant will prevent lytic reactivation and replication (necessary). Although other rhadinovirus mRNAs are transcribed in the presence of cycloheximide (CHX) (Orf57/Mta, K8/Zta, Orf45) and are therefore considered immediate-early genes, their gene products are not sufficient to reactivate virus from latency. Whether any of these are necessary for lytic replication remains to be established.

Figure 1 shows an array analysis of RRV transcription at 6 h after de novo infection in the presence or absence of permissive fibroblasts (from Dittmer et al. 2005). Here, the levels for each viral mRNA were measured by quantitative real-time RT-PCR, and for each viral mRNA the number of cycles that were required to obtain a fixed amount of product was plotted. Rta/Orf50 is the most abundant mRNA at early times after infection and is unaffected by CHX. In contrast, the majority of RRV transcripts are not transcribed that early in the infection process (requiring more than 40 cycles of PCR to detect a signal under either condition) or are significantly inhibited by CHX. The latter includes mRNAs driven by Rta/Orf50-responsive promoters. But there are also a significant number of mRNAs that were transcribed in the presence of the protein synthesis inhibitor. By definition these are immediate-early genes, and their transcription is dependent only on preformed (i.e., immediate early) cellular regulators or RRV virion transactivators. Yet at the same time many of these genes are also Rta/Orf50-responsive. Rta/Orf50's own promoter falls into this class. In the case of the Rta/Orf50 promoter, transactivation by Rta/Orf50 protein establishes a direct positive feedback loop that locks the lytic transcription cascade into place. On the basis of extensive transcriptional profiling, we would speculate that the gamma herpesviridae evolved a more plastic, less ridged transcriptional control program than the alpha and beta herpesviridae to cope with the various signaling events, cytokine exposures, and growth stimuli in the life of a latently infected lymphocytes.

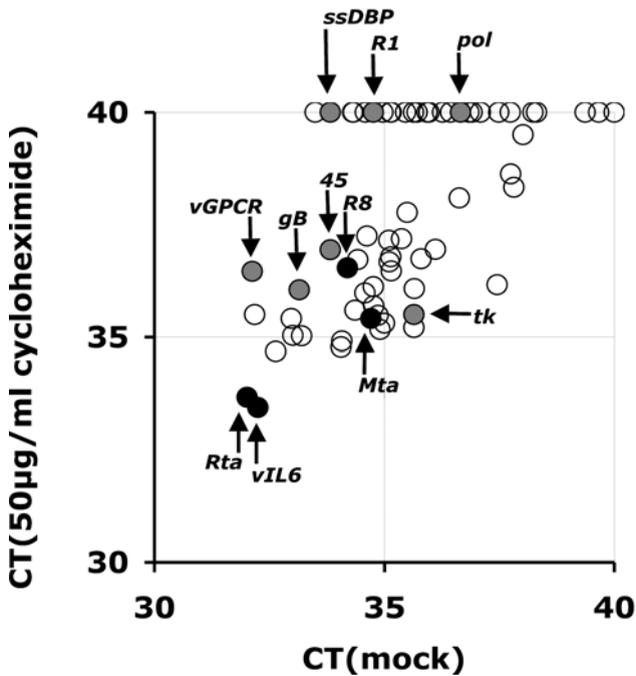


Fig. 1 Array analysis of RRV transcription at 6 h after de novo infection in the presence (*vertical*) or absence (*horizontal*) of cycloheximide in permissive fibroblasts (from Dittmer et al. 2005). Shown are relative mRNA levels on a log₂ scale (*CT*). Known KSHV immediate-early genes are in *black*, known Rta targets in KSHV in *gray*, and all others in *open circles*

3

Lytic Reactivation

All gammaherpesviruses encode an Rta/Orf50 homologue, and each has been shown to play a pivotal role in the initiation of viral lytic gene expression and lytic reactivation from latency. Although the gene product of *Orf50*, named Rta (replication and transcription activator), is the only essential latent/lytic switch protein for the gamma-2-herpesviruses (rhadinoviruses), two proteins, Zta and Rta, independently can reactivate EBV, a gamma-1 or lymphocryptovirus, from latency. This difference in viral lytic switch proteins between the lymphocryptoviruses and rhadinoviruses indicates a marked difference in the precise molecular mechanisms of virus-mediated lytic reactivation of the two related subgroups of gammaherpesviruses.

3.1

Lymphocryptovirus—EBV

Many excellent reviews exist that describe EBV lytic reactivation in detail. Hence, we will only recount the basic tenets here to compare them vis-à-vis rhadinovirus lytic reactivation. The major viral lytic switch protein of EBV is considered to be Zta (also known as Zebra, *BZLF-1*, or Z protein) (Chen et al. 1999; Cox et al. 1990; Quinlivan et al. 1993; Ragozy et al. 1998; Zalani et al. 1996). Zta is a bZIP-type transcriptional transactivator. The EBV Orf50 homologue, the *BRLF1* gene product Rta, is also a sequence-specific DNA-binding protein known to function as a transcriptional activator (Quinlivan et al. 1993; Ragozy et al. 1998; Ragozy and Miller 2001; Russo et al. 1996). Independently, both Zta and Rta initiate the expression of lytic genes and, in a somewhat cell type-specific manner, Rta can lead to the activation of DE promoters. It is believed that Zta and Rta proteins of EBV act synergistically (although Zta can suppress the transactivation function of Rta) to induce viral reactivation in latently infected B cells. However, EBV Rta alone can activate a subset of lytic promoters that do not require Zta, and EBV Zta alone can activate a subset of lytic promoters that do not require Rta (Ragozy and Miller 1999). A deletion mutant of either Zta or Rta is defective for viral reactivation (Feederle et al. 2000). The KSHV Rta/Orf50 is the sequence homologue of the EBV Rta/BRLF1, whereas the KSHV KbZIP/K8 protein is the sequence homologue of EBV Zta/BZLF1. In contrast to EBV, the KSHV Rta/Orf50 is considered to be the only essential lytic switch protein to date. A KSHV Rta/Orf50 deletion mutant is incapable of reactivation from latency (Xu et al. 2005), although a deletion mutant of KSHV KbZIP remains to be evaluated. In this regard the two subclasses of gammaherpesviruses (lymphocryptovirus and rhadinovirus) differ in their molecular mechanisms of reactivation in that to date only one viral protein, Rta/Orf50, has been shown to mediate viral reactivation and induce lytic gene expression in the rhadinoviruses (KSHV, HVS, RRV, and MHV-68). In fact, expression of KSHV Rta/Orf50 precedes expression of K8 (K-bZip) and transactivates the K8 promoter (Lukac et al. 1998; Sun et al. 1998). Therefore, in an effort to minimize complexity and derive general principles for homologous molecular mechanisms, we will only discuss the Rta/Orf50 proteins of the rhadinoviruses.

4

Rhadinoviruses—KSHV, HVS, RRV, and MHV-68

4.1

Experimental Considerations

Before we delve into a detailed molecular description of the rhadinovirus Rta/Orf50 transactivator, it seems prudent to highlight some experimental constraints that affect the general conclusions we can draw from the many studies on Rta/Orf50 and rhadinovirus reactivation. KSHV, RRV, and MHV68 establish latency in B lymphocytes and are associated with B-cell hyperplasia, but unlike EBV and primate lymphocryptoviruses, KSHV, RRV, and MHV68 do not immortalize primary B cells with any efficiency in culture. Rta/Orf50 is sufficient to reactivate latent virus in each case, but the exact B-cell compartment and lineage-specific transcription factor makeup may be different for each virus. Only RRV and MHV68 establish a robust *de novo* infection in primary fibroblasts, which amplifies input virus. Hence, Rta/Orf50's role in primary infection under low MOI conditions can only be investigated in these lytic model systems. KSHV infects primary and immortalized endothelial cells, but to date only a single plaque has been published (Boshoff et al. 1995; Ciuffo et al. 2001). Although KSHV can be serially propagated, input virus is not amplified and high MOI infection in the presence of polybrene is required to initiate the culture (Foreman et al. 1997; Lagunoff et al. 2002; Renne et al. 1998). Reactivation from latency in response to biological signals or chemical inducers such as phorbol ester is a low-frequency event for KSHV (Chang et al. 2000; Renne et al. 1996) and sets up an interesting paradox. In a latent culture every single cell is infected with KSHV, carries 10–50 copies of the viral episome, and expresses the latency-associated nuclear antigen (LANA/Orf73). LANA is necessary and sufficient for latent viral replication, which is analogous to Rta/Orf50's requirement for lytic viral replication (Ballestas et al. 1999; Godfrey et al. 2005). If a latent cell culture, for instance, BCBL-1 cells, is treated with phorbol ester, 100% of the cells receive the drug and are subject to drug action; however, not all cells will express Rta/Orf50, and of the cells that express Rta/Orf50 not all cells express delayed-early genes. Furthermore, within the subset of cells that express delayed-early genes even fewer yet express true late viral mRNAs, such as Orf29, a capsid component (Zoetewij et al. 1999). Therefore, we speculate that additional constraints exist that regulate viral replication at each junction (IE, DE, E, L) in the regulatory cascade.

McAllister et al. showed that the cell cycle state influences how competent each host cell is to support viral replication (McAllister et al. 2005). At this point it is unresolved whether the host cell cycle state simply reflects responsiveness of the PKC downstream targets that activate the Rta/Orf50

promoter, the effectiveness of Rta/Orf50 to function as a transactivator, or steps dependent on viral DE genes. Alternatively, cells in S phase may remain viable for a longer time and can, therefore, accumulate more viral mRNA and proteins before dying because of the effects of viral capsid maturation and egress. Many cell lines do not tolerate stable Rta/Orf50 expression, but recently exciting new tools have become available to investigate Rta/Orf50 function and add to the existing tools of cDNA expression (Sun et al. 1998; Lukac et al. 1998) and Rta/Orf50 dominant-negative plasmids (Lukac et al. 1998): an *Orf50* k/o virus for MHV68 and KSHV, a KSHV-inducible system in BCBL-1 and 293 cells (Nakamura et al. 2003; Xu et al. 2005), a KSHV Rta/Orf50 recombinant adenovirus (Liang et al. 2002), and novel DNA-binding mutants of KSHV Rta/Orf50 (Chang et al. 2005). These tools will yield exciting new insights into the molecular function of Rta/Orf50 and the biology of the rhadinoviruses, although they may also lead to discrepancies with existing studies due to differences in the experimental approach.

4.2

Chemically Induced Viral Reactivation of Gammaherpesviruses

Reactivation of rhadinoviruses in latently infected cells can be achieved by treatment with chemicals that mimic BCR signaling such as *n*-butyrate, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or calcium ionophores. These types of treatment lead to expression of viral lytic genes, foremost among them *Orf50*. Although the entire cellular signaling cascades that lead to viral reactivation in response to these chemical treatments are unknown, we do know that induction of the KSHV *Orf50* promoter by TPA involves the cellular AP1 pathway and induction by sodium butyrate (NaB) involves cellular Sp1 (Wang et al. 2004a, 2003a, 2003b). Cannon et al. have recently shown that overexpression of the KSHV lytic protein vGPCR/Orf74 indirectly resulted in a decreased efficiency of chemical induction of the ORF50 transcript (Cannon et al. 2006). Because the KSHV vGPCR/Orf74 is known to modulate cellular signaling pathways, which, in extreme instances, can lead to transformation (Bais et al. 1998; Polson et al. 2002) this adds credence to a model of multilayered cross talk between KSHV and the host. In RRV, the histone deacetylase inhibitor *trichostatin A* (TSA) is also capable of reactivating RRV from latently infected cells, presumably by de-repressing the *Orf50* promoter (DeWire et al. 2002). Like TSA and butyrate, valproic acid (2-propylpentanoic acid) also has potent histone deacetylase activity. At the same time it is FDA approved as antiseizure medication. Treatment of KSHV-infected primary effusion lymphoma (PEL) cells with valproate induced lytic reactivation in culture (Shaw 2000; Klass et al. 2005) and is currently in clinical trials for the treatment of Kaposi's sarcoma.

4.3

Viral Induction of Lytic Reactivation of Rhadinoviruses

Although many viral proteins were assayed for their ability to reactivate KSHV in PEL cells, only ectopic expression of Rta/Orf50 was sufficient to disrupt latency and activate lytic replication, resulting in a complete productive viral life cycle (Gradoville et al. 2000; Lukac et al. 1998; Sun et al. 1998). The KSHV Zta and Mta homologues by themselves were not able to induce lytic reactivation, although, as in EBV, they may transactivate some DE promoters independently of Rta/Orf50. Expression of HVS Orf50a protein and MHV-68 Rta/Orf50 also induces lytic reactivation and production of infectious viral progeny in HVS and MHV-68 models of latency, respectively (Goodwin et al. 2001; Wu et al. 2000). The RRV Rta/Orf50 and MHV-68 Rta/Orf50 can reactivate KSHV from latency (Damania et al. 2004), and the KSHV Rta/Orf50 protein can reactivate the murine viral homologue, MHV-68, from latency (Rickabaugh et al. 2005). These studies demonstrate a strong conservation of function across evolution of the rhadinovirus Rta proteins. Although RRV Orf50 has been shown to be a potent transactivator of RRV DE promoters, it has not yet been demonstrated to reactivate RRV from latency (DeWire et al. 2002), because lytic replication rather than latency is the default pathway following primary RRV infection of cells in culture. Recently, a latently infected RRV system has been established, and we can expect formal demonstration of this in the near future (DeWire and Damania 2005).

5

Rta/Orf50 Transcription

The Orf50 transcripts from all gamma-2-herpesviruses share a similar architecture that is essentially comprised of two exons separated by one intron. A major IE transcript is observed after reactivation of KSHV that is a 3.6-kb tricistronic mRNA encoding the Orf50, K8, and K8.1 reading frames (Zhu et al. 1999). Splicing results in the major Orf50 transcript; however, other alternatively spliced mono-, bi-, and polycistronic Orf50-containing transcripts have been found (Saveliev et al. 2002; Tang and Zheng 2002; Wang et al. 2004). Presently, the significance of these isoforms is unclear. Splicing of the Orf50 transcript is a characteristic of gamma-2-herpesviruses and is in contrast to the Orf50 cDNA of the gamma-1 herpesvirus EBV, which is identical to its genomic open reading frame structure (Manet 1989). In the rhadinoviruses, Orf45 is located within the Rta/Orf50 intron in opposite orientation and the rhadinovirus Orf45 promoter is presumably located within the Rta/Orf50 open reading frame. Similarly in EBV, Orf45 (BRRF1/Na) is located 5' of the

Rta/Orf50 open reading frame, also in the opposite orientation and also within the first intron (the first exon of EBV Rta is noncoding) (Hong et al. 2004).

The major 3.6-kb Orf50 transcript is induced within 4 h of *n*-butyrate treatment of latently infected PEL cells and is resistant to treatment with the protein synthesis inhibitor, cycloheximide (CHX), thus displaying IE kinetics (Sun et al. 1999; Zhu et al. 1999). Lukac et al. also observed expression of Orf50 mRNA within 1 h of inducing viral reactivation in latently infected BCBL-1 cells by treatment with the phorbol ester TPA (Lukac et al. 1998). MHV-68 *Orf50* is also an immediate-early gene, as is the RRV *Orf50*, further demonstrating conservation of *Orf50* transcription kinetics among the rhadinoviruses (Rochford et al. 2001; DeWire et al. 2002; Dittmer et al. 2005). Of the rhadinoviruses, lytic reactivation of HVS results in transcription of two distinct Orf50 mRNA species, called *Orf50a* and *Orf50b* (Whitehouse et al. 1997). The Orf50a transcript is identical to that described for the other rhadinoviruses and is detected at early times during viral replication. The HVS Orf50b transcript is expressed at later time points during replication and is produced from a promoter within the second exon. Its function remains to be elucidated. Because abolishing Rta/Orf50 function inhibits lytic replication at IE times, it has not been possible to determine experimentally whether Rta/Orf50 has additional functions at DE or late times in any of the rhadinoviridae.

5.1

Regulation of the KSHV Rta/Orf50 Promoter

Because Rta/Orf50 protein is the key regulator of KSHV lytic reactivation, much attention has focused on the promoter that regulates Rta/Orf50 expression. The KSHV Rta/Orf50 protein autoregulates its own promoter via an indirect mechanism, because no obvious Rta/Orf50-responsive element (RRE) or RBP-J- κ consensus binding sites are present within the *Orf50* promoter (these mechanisms are described in detail in later sections of this review) (Chang and Miller 2004; Deng et al. 2000; Gradoville et al. 2000). By comparison to other Rta/Orf50-responsive promoters, the *Orf50* promoter is only marginally activated by Rta/Orf50 protein expression. Within the *Orf50* promoter, a binding sequence for the cellular transcription factor octamer-binding protein (Oct-1) was shown to mediate autoregulation by Rta/Orf50 protein. Oct-1 bound to a specific region of DNA within the *Orf50* promoter, as demonstrated by electrophoretic mobility shift assay (EMSA) (Sakakibara et al. 2001). In addition, both Sp1 and Sp3 cellular transcription factors appear to be involved in Rta/Orf50 autoactivation (Chen et al. 2000; Zhang et al. 1998). This is similar to the EBV Rta protein, which also mediates autoregulation of

its own promoter (Rp) via a non-DNA-binding mechanism involving cellular Sp1 and Sp3 proteins (Ragoczy and Miller 2001). KSHV Rta/Orf50 also interacts with the CCAAT/enhancer binding protein α (C/EBP- α) to upregulate Rta/Orf50 expression (Wang et al. 2003b).

The Orf50 promoter is heavily methylated in latently infected PEL cells, and treatment with TPA leads to demethylation of the promoter (Chen et al. 2001). In vivo, several biopsies from KSHV-related diseases, including Kaposi's sarcoma, multicentric Castleman disease (MCD), and PEL, showed decreased methylation of the *Orf50* promoter, although this promoter was still heavily methylated in samples obtained from a latently infected KSHV carrier. This evidences an additional layer of regulation of Rta/Orf50 expression and, as such, KSHV lytic reactivation. Progressive methylation of viral lytic promoters could skew KSHV infection of host cells toward latency. This is consistent with the decoration of the viral episome and Rta/Orf50 promoter with inhibitory histone complexes (Lu et al. 2003). Because much of the investigation of Rta/Orf50's function is based on transient transfection assays of unmethylated, histone-free promoter-reporter plasmids, we do not know how Rta/Orf50's *general* transactivation function (via TAFs and regulation of HDACs) may work together with Rta/Orf50's *specific* transactivation functions (via direct DNA binding or RBP-J- κ binding sites).

The propensity of KSHV to establish latency on primary infection (Ciufu et al. 2001; Grundhoff and Ganem 2004; Krishnan et al. 2004; Moses et al. 1999) is in contrast to the propensity of RRV and MHV-68 to establish lytic infection. The bias of KSHV toward the establishment of latency after primary infection may be due to Rta/Orf50's interaction with species-specific chromatin modules, because Rta/Orf50 proteins of any rhadinovirus are able to transactivate unmethylated viral promoters in transient transfection assays. However, it is interesting to note that both MHV-68 and RRV are impaired in their ability to reactivate KSHV in the context of viral gene expression (Damanian and Jung 2001).

Micrococcal nucleosome mapping techniques by Chen et al. reported a nucleosome positioned on the Orf50 promoter that overlapped the transcription start site and a GC-rich region bound by both Sp1 and Sp3 (Lu et al. 2003). The Sp1/Sp3 region of the Orf50 promoter was also mapped as being highly responsive to two chemical compounds known to inhibit histone deacetylases, NaB and TSA (Lu 2003). In addition, NaB treatment led to the rapid recruitment of In11/Snf5, a component of the Swi/Snf family of chromatin remodeling proteins. These data describe complex, multitiered levels of transcriptional regulation of the Orf50 promoter within latently infected host cells.

6

Rta/Orf50 Protein

6.1

KSHV Rta/Orf50

The KSHV *Orf50* gene encodes a 691-amino acid protein that is highly phosphorylated and localizes to the nucleus of mammalian cells (Lukac et al. 2001, 1999; Seaman and Quinlivan 2003). All Rta homologues share a conserved C-terminal activation domain. Deletion of 160 amino acids in the C-terminal activation domain of the KSHV Rta/Orf50 results in production of a truncated, but stable, Rta/Orf50 protein that forms multimers with wild-type Rta/Orf50 in PEL cells and functions as a dominant-negative inhibitor of Rta/Orf50 transactivation (Lukac et al. 1998). Expression of this truncated Rta/Orf50 protein leads to suppression of both spontaneous and chemically induced viral reactivation. Expression of KSHV Rta/Orf50 and subsequent viral reactivation can also be efficiently knocked down by expression of human RNase P (Zhu et al. 2004). These data suggest that Rta/Orf50 is, indeed, the lytic switch protein of KSHV. Recently, Pari and colleagues reported genetic evidence for the Rta-lytic switch hypothesis by utilizing the KSHV genome cloned into a bacterial artificial chromosome (BAC36) (Gao et al. 2003; Zhou et al. 2002) and generating a deletion within the *Orf50* open reading frame (Xu et al. 2005). After transfection of the *Orf50*-deficient BAC into HEK 293 cells, latent genes were expressed at wild-type levels; however, the virus was unable to reactivate on chemical treatment, unequivocally demonstrating that Rta/Orf50 is required for successful viral reactivation.

A provocative recent report showed that Rta/Orf50 was present in KSHV virions (Bechtel et al. 2005b), which would make it a virion transactivator much like the herpes simplex virus VP16 and thus ensure lytic replication on primary infection. However, herpesvirus virions are notorious for capturing a variety of proteins, and even mRNAs (Bechtel et al. 2005a), simply as scaffolds during assembly or because of sloppy egress. The composition of nonstructural proteins in the rhadinovirus virions is highly variable (Zhu and Yuan 2003; Zhu et al. 2005; O'Connor and Kedes 2006; Trus et al. 2001) and may not necessarily have a function in the next infection cycle.

The different functions of KSHV Rta/Orf50 are subject to posttranslational modifications. KSHV Rta/Orf50 is highly phosphorylated, a modification that is mediated at least in part by the ability of Rta/Orf50 to bind to and be phosphorylated by the cellular Ste20-like kinase hKFC (Gwack et al. 2003b; Lukac et al. 2001, 1999). The phosphorylation of Rta/Orf50 by hKFC as well as Rta's poly(ADP)-ribosylation by cellular PARP-1 protein both result in decreased ability of KSHV Rta/Orf50 to transactivate viral promoters (Gwack

et al. 2003b). Recently, KSHV Rta/Orf50 was shown to have E3 ubiquitin ligase activity and could direct polyubiquitination of cellular interferon regulatory factor 7 as well as polyubiquitination of itself (Yu et al. 2005). Point mutations in the Cys + His-rich N-terminal domain of Rta/Orf50 abolished the E3 ligase activity, which should be independent of Rta's transcriptional activity. Much more still needs to be learned, but the many posttranslational modifications of KSHV Rta/Orf50 most likely act to regulate the function, and possibly the stability, of this key viral transactivator protein.

KSHV Rta/Orf50 has been shown to modulate transcription of host genes as well. It has been reported to transactivate cellular interleukin 6 (IL-6) transcription (Deng et al. 2002a). KSHV Rta/Orf50 can also modulate the ability of cellular STAT3 to function as a transactivator, indirectly leading to modulation of host gene expression (Gwack et al. 2002). Furthermore, Rta/Orf50 interacts with RBP-J- κ (Chang et al. 2005) and may thereby regulate the transcription of RBP-J- κ -dependent host mRNAs.

6.2

MHV-68 Rta/Orf50

The Rta protein encoded by *Orf50* of MHV-68 is sufficient to induce lytic reactivation in latently infected cells (Wu et al. 2000). Investigations of the disruption of the MHV-68 Rta/Orf50 open reading frame demonstrated that Rta/Orf50 is also necessary for viral reactivation (Liu et al. 2000; Pavlova et al. 2005, 2003). The requirement for MHV-68 Rta/Orf50 in lytic reactivation was also demonstrated by other means, whereby a loss in viral reactivation was observed after efficient knockdown of MHV-68 Rta/Orf50 expression by RNAi (Jia et al. 2004). A mutant MHV-68 virus, called M50, was generated to constitutively express MHV-68 Rta/Orf50 by insertion of a new promoter element into the 5'-untranslated region (UTR) of the Orf50 promoter (May et al. 2004). Constitutive expression of Rta/Orf50 by the M50 mutant MHV-68 virus resulted in defective establishment of latency. The physiological relevance of this mutant phenotype was demonstrated by studies in which immunization of mice with the mutant M50 virus resulted in partial protection against challenge with wild-type virus, demonstrating the importance of proper transcriptional regulation of *Orf50* in the both the pathogenesis and establishment of latency by MHV-68 (Boname et al. 2004; May et al. 2004). Importantly, a Rta/ORF50-null mutant of MHV-68 established long-term latency in the lungs of infected mice but failed to vaccinate against a wild-type virus challenge, therefore implicating the necessity of lytic replication for generation of a protective immune response (Moser et al. 2006). In addition, gene array studies of a recombinant virus that overexpressed MHV-68 Orf50

found that nearly every MHV-68 gene assayed was upregulated by Rta/Orf50 overexpression. This phenotype is consistent with Rta/Orf50 being the first gene in the MHV-68 lytic transcriptional cascade and highlights the potent transactivating ability of the protein (Martinez-Guzman et al. 2003).

6.3

HVS Rta/Orf50

Much less is known about HVS Rta/Orf50, but what has been reported suggests that the properties of HVS Rta/Orf50 resemble those of the other rhadinoviruses. HVS Rta/Orf50 induces viral reactivation of latently infected cells (Goodwin et al. 2001), a function conserved throughout all rhadinoviruses. HVS Rta/Orf50 contains an AT-hook DNA binding domain that is required for transactivation of at least two delayed early viral promoters, Orf6 and Orf57 (Walters et al. 2004). Although this report demonstrates that HVS Rta/Orf50 can transactivate certain viral promoters via direct DNA binding, this viral transactivator most likely also works through indirect mechanisms as do the lytic switch proteins of the other gammaherpesviruses. HVS Rta was shown to bind TATA-binding protein (TBP) *in vitro*, which provides an alternative means to influence viral and cellular transcription (Hall et al. 1999). Of note, the amino acid sequence of both Rta/Orf50 isoforms, Orf50a and Orf50b of HVS A11 and HVS C488 (a low-passage transforming isolate), are among the most divergent open reading frames in these viral isolates (Ensser et al. 2003), with only ~70% amino acid identity compared to >90% amino acid identity for the other 60 of 75 (80%) open reading frames in the HVS genome. Whereas the Orf50b of A11 showed decreased transactivation capability compared to Orf50a of A11, the Orf50b of C488 demonstrated full transactivation capability. In contrast, both the A11 and C488 Orf50a proteins were capable of reactivating lytic replication in persistently infected cells, indicating a conservation of function for one Rta/Orf50 isoform and not the other. The same study reported that Rta/Orf50 allelic variation cosegregated with Stp and Tip in their ability to transform human T cells in culture. By inference based on this genetic data set alone, Rta/Orf50 could be considered an oncogene. On the other hand, there is no independent evidence that Rta/Orf50 can transform cells in culture or can cooperate with other oncogenes to do so. Rather, long-term stable expression of Rta/Orf50 seems incompatible with continued cell growth. No molecular mechanism has been described to support such classification of HVS Rta/Orf50 as an oncogene, but one can imagine that a promiscuous transactivator like Rta/Orf50 could reset the cellular transcription profile via interactions with RPB-J- κ or other via other mechanisms. This was recently explored by Nakamura et al. using a tetracycline-inducible

KSHV Rta/Orf50 cell line (Chang et al. 2005). Among the host genes that were induced by Rta/Orf50 via RPB-J- κ were CD21 and CD23a, which are involved in lymphocyte activation. KSHV Rta/Orf50 also inhibits the p53 tumor suppressor protein (Gwack et al. 2001b) and this capability is conserved among the primate rhadinoviruses, though not in MHV-68 (Damania et al. 2004). Overall, Rta/Orf50 is a key regulator of both viral and cellular transcription. An in-depth analysis of the molecular mechanisms of transactivation by Rta/Orf50 is described below.

7

Rta/Orf50 Function

7.1

Viral Promoters Transactivated by KSHV Rta/Orf50

KSHV Rta/Orf50 is a strong transactivator of many viral promoters in transient transfection assays. A summary of these promoters is reported in Table 1.

7.2

Viral Promoters Transactivated by MHV-68 Rta/Orf50

Ectopic expression of MHV-68 Rta/Orf50 in latently infected cells leads to expression of lytic proteins (as determined by Western blots of cell extracts probed with immune mouse serum) and is sufficient to drive viral reactivation of latently infected cells (Wu et al. 2001, 2000). The MHV-68 Orf57 promoter is transactivated by Rta/Orf50. MHV-68 RREs are found within the Orf57 promoter region of the viral genome, as they are in the KSHV Orf57 promoter (Pavlova et al. 2005). In addition, the MK3 promoter was reported to contain an MHV-68 Rta-responsive element (Coleman et al. 2003). Potential MHV-68 RREs bear significant homology to published KSHV RREs, but to date fewer MHV-68 promoters than KSHV promoters have been investigated in detail.

7.3

Viral Promoters Transactivated by RRV Orf50

RRV Orf50 has also been shown to transactivate promoter-reporter constructs in transient transfection assays. The R8, Orf57, and gB promoters of RRV were highly activated and the vIRF promoter was only slightly activated by Rta/Orf50 (DeWire et al. 2002; Lin et al. 2002). In contrast to KSHV, RRV Rta/Orf50 did not autoactivate its own promoter.

Table 1 Viral promoters transactivated by KSHV Rta/Orf50

KSHV viral promoter	Mechanism	Reference
Nut-1/PAN	C/EBP- α , DNA	Song et al. 2001; Wang et al. 2003b
Kaposin (K12)	DNA	Lukac et al. 1998; Song et al. 2003
K-bZip (K8)	Ap-1, DNA	Lukac et al. 1999; Wang et al. 2003a, 2004
MTA (ORF57)	RBP-J- κ , C/EBP- α , DNA	Duan et al. 2001; Liang et al. 2002; Lukac et al. 1999; Wang et al. 2003b
K6 (vMIP-1)	RBP-J- κ	Chang et al. 2005
K5	Unknown	Haque et al. 2000
K1	Unknown	Bowser et al. 2002
vIRF (K9)	Sp1	Chen et al. 2000; Ueda et al. 2002
ssDNA-binding (ORF6)	RBP-J- κ	Liang et al. 2002
DNA pol.-pross (ORF59)	Unknown	Nishimura et al. 2001
Thymidine kinase (ORF21)	Sp1	Zhang et al. 1998
vOX-2 (K14)	RBP-J- κ	Jeong et al. 2001; Liang and Ganem 2004
vGPCR (ORF74)	RBP-J- κ	Jeong et al. 2001; Liang and Ganem 2004
vIL-6 (K2)	DNA	Deng et al. 2002
LANA LT _i	RBP-J- κ	Matsumura et al. 2005; Lan et al. 2005; Staudt and Dittmer, 2006
Rta (ORF50)	Oct-1, Sp1, Sp3, C/EBP- α	Chen et al. 2000; Deng et al. 2000; Gradoville et al. 2000; Sakakibara et al. 2001; Wang et al. 2003a; Zhang et al. 1998

7.4

Viral Promoters Transactivated by HVS Orf50

The HVS Rta/Orf50a does transactivate its own promoter, as well as the DE Orf6, Orf57, and Orf9 viral promoters of HVS (Whitehouse et al. 1997; Thureau

et al. 2000; (Walters et al. 2005, 2004; Byun et al. 2002). The HVS Rta/Orf50a contains an AT-hook DNA binding domain that is required for transactivation of the Orf6 and Orf57 promoters, and cellular C/EPB- α synergizes with HVS Rta/Orf50a to transactivate the Orf9 DNA polymerase promoter (Walters et al. 2004). HVS Rta/Orf50a was found to autoregulate its own promoter by use of a 36-bp RRE that has no significant homology to previously reported RREs of any of the gammaherpesviruses. The HVS RRE DNA sequence conferred Rta/Orf50a responsiveness to an enhancer-less SV40 minimal promoter (Walters et al. 2005).

8 Mechanisms of Rta/Orf50 Transactivation

8.1 KSHV Orf50-Responsive Elements and Direct DNA Binding

There have been many reports showing data that KSHV Rta/Orf50 protein transactivates viral promoters by direct DNA binding to sequences found within these promoters [termed Rta response elements (RREs) or Orf50 response element in n promoter ($50RE_n$)]. KSHV Rta/Orf50 can transactivate two delayed-early (DE) promoters, Orf57 and K8 (K-bZip), by direct DNA binding (Lukac et al. 2001; Duan et al. 2001; Song et al. 2002). The N-terminal 272 amino acids of Rta protein are sufficient to bind a 12-bp DNA sequence, 5'-AACAAATAATGTT-3', found within both DE promoters and termed the $50RE_{57}$. KSHV Rta/Orf50 also transactivates the PAN/nut-1 and K12 promoters (Chang et al. 2002) and was shown to directly bind DNA within these promoters. Intriguingly, the sequences of the $50RE$ found within the Pan/nut-1 and K12 promoters (5'-AAATGGGTGGCTAACCCCTACATAA-3', PAN DNA sequence shown, K12 promoter sequence underlined) and the $50RE_{57}$ (5'-AACAAATAATGTT-3') share no significant homology. Yet another Orf50-responsive element was discovered in the vIL-6 promoter that contains a 26-bp sequence, 5'-AAACCCCGCCCCCTGGTGCTCACTTT-3' (Deng et al. 2002b). Direct comparison of RRE-containing viral promoters revealed that the transcription initiation rate of these promoters, as opposed to transcript stability, is the major determinant of expression of these viral proteins (Song et al. 2003). Liao et al. reported that KSHV Rta/Orf50 forms oligomers and makes multiple contacts with a tandem array of phased A/T triplets in the configuration of (A/T)₃ (G/C)₇ repeats (Liao et al. 2003a). An RRE and TATA box was also found within the KSHV OriLyt, and this DNA region functioned as an Rta/Orf50-responsive promoter when cloned into a reporter vector (Wang et al. 2004b). This promoter regulated a late 1.4-kb polyadenylated mRNA that

was sensitive to the viral DNA polymerase inhibitor *foscarnet* and has coding capacity for a 75 aa open reading frame whose gene product is of unknown function.

HMGB1 is a cellular protein belonging to the high-mobility group (HMG) box protein subfamily that affects transactivation function of both EBV Zta and EBV Rta (Ellwood et al. 2000; Mitsouras et al. 2002). HMG proteins are large chromosomal proteins thought to function to promote higher-order DNA-protein complexes by changing DNA conformation to be more easily accessible to transcriptional machinery. HMGB1 was recently shown to enhance direct DNA binding of KSHV Rta/Orf50 to RREs in vitro and to enhance transactivation functions of both KSHV and MHV-68 Rta/Orf50 proteins in transient transfection assays (Song et al. 2004).

KSHV Rta/Orf50 was reported to contain a protein domain that seemed to act in an autoregulatory fashion. This autoregulatory domain of KSHV Rta/Orf50 is contained within amino acids 521–534 and functions to control the direct DNA binding ability of the protein as well as protein stability (Chang and Miller 2004). Deletion of amino acids 521–534 or mutation of a basic motif (KKRK) at aa 527–530 dramatically enhanced DNA binding of Rta/Orf50. Although the DNA binding ability of the KKRK mutant was enhanced, its ability to transactivate the PAN promoter was impaired, suggesting that these two functions do not correlate synergistically on the PAN promoter. In addition, expression of autoregulation-domain mutants led to appearance of an alternative form of Rta, termed Orf50b, which showed decreased posttranslational modifications. At this time, investigations into the structure and posttranslational modifications of KSHV Rta/Orf50 are in the early stages, and we can expect more insights in the near future.

8.2

Interaction of Rta/Orf50 with RBP-J- κ

Although KSHV Rta/Orf50 binds to DNA containing the RREs described above, no obvious consensus sequence was found among other Rta/Orf50-responsive promoters: an observation that prompted many to look for an alternate mechanism of Rta transactivation. Ganem and colleagues used a yeast-two-hybrid approach to assay possible cellular binding proteins and found that the cellular protein RBP-J- κ (also called CSL or CBF-1) interacted with KSHV Rta/Orf50 (Liang et al. 2002). RBP-J- κ is a sequence-specific DNA binding protein and is the downstream effector of Notch signal transduction (Mumm and Kopan 2000). In uninfected cells RBP-J- κ functions as a transcriptional repressor until ligand-mediated Notch signaling occurs, which leads to the conversion of RBP-J- κ from a repressor to a transactivator of down-

stream cellular gene targets (such as HES, hairy and enhancer of split genes). KSHV can usurp the function of cellular RBP-J- κ without the requirement for Notch-ligand interaction, as the binding of KSHV Rta/Orf50 protein to RBP-J- κ converts RBP-J- κ from a transcriptional repressor to a transactivator. This mechanism has been demonstrated for the KSHV Mta/Orf57, SSB/Orf6, PAN/nut-1, vGPCR/K14, vMIP-1/K6, and LT_i promoters (Liang et al. 2002; Liang and Ganem 2003, 2004; Lan et al. 2005; Matsumura et al. 2005; Staudt and Dittmer 2006). Amino acids 170–400 of Rta/Orf50 mediate binding to RBP-J- κ , and there are two contiguous but distinct regions of RBP-J- κ to which Rta/Orf50 binds: one is within the central repressor domain and one is within the N-terminal domain of RBP-J- κ . It is striking that the central repressor domain of RBP-J- κ to which KSHV Rta/Orf50 binds is the same region to which Notch, the physiological effector protein of RBP-J- κ , binds as well. This suggests that Rta/Orf50 replaces Notch during lytic reactivation in B cells; however, this has yet to be demonstrated. Liang and Ganem propose that KSHV may employ the repressive function of RBP-J- κ bound to lytic promoters as a means of maintaining latency in the absence of appropriate reactivation stimuli (Liang and Ganem 2003). The interaction between KSHV Rta/Orf50 and cellular RBP-J- κ demonstrates an elegant mechanism the virus has developed to hijack an essential cellular signal transduction pathway as a means to obtain control over viral latency and lytic reactivation.

8.3

Interaction of Rta/Orf50 with Other Cellular Transcription Factors

The ubiquitously expressed cellular transcription factor Sp1 plays an important role in Rta/Orf50 transactivation of promoters, although to date there have been no reports of direct binding between KSHV Rta/Orf50 and cellular Sp1 protein. Sp1 binding sites within the *Orf50* promoter are essential for butyrate-induced Rta/Orf50 expression and lytic replication (Ye et al. 2005). Sp1 is also involved in Rta/Orf50 transactivation of other viral promoters, including vIRF/K9, thymidine kinase/Orf21, and Rta/Orf50 (Chen et al. 2000; Ye et al. 2005; Zhang et al. 1998).

Using a proteomics approach, Wang et al. identified a novel cellular protein, MGC2663, that stably bound to Rta/Orf50 after tandem immunoaffinity chromatography (Wang et al. 2001). MGC2663 was found to bind KSHV Rta/Orf50 and specifically synergized with Rta/Orf50 to activate viral transcription. The MGC2663 protein was previously uncharacterized, but Wang et al. found that it was expressed in every primate cell line tested and that it enhanced transactivation by Rta/Orf50, hence assigning MGC2663 the name *K-RBP* for KSHV Rta binding protein.

KSHV Rta/Orf50 also binds CBP [cyclic AMP (cAMP)-responsive element binding protein (CREB)-binding protein], which is a transcriptional coactivator that contains intrinsic histone acetyltransferase (HAT) activity (Gwack et al., 2003a, 2001a). Acetylation of histones is associated with relaxing nucleosomal structures, thus rendering regions of tightly packed DNA open and accessible to the transcriptional machinery. Binding of CBP to KSHV Rta/Orf50 increased the ability of Rta/Orf50 to transactivate viral promoters. Binding between these two proteins is mediated by the N-terminal basic domain of Rta/Orf50, which contains a conserved LxxLL CBP-binding motif, and the C/H3 domain and C-terminal transactivation domain of CBP. In addition, other cellular CBP-binding proteins, including CBP-BP and c-Jun, enhanced the ability of Rta/Orf50 to transactivate viral promoters. The transactivation function of EBV Rta is also enhanced by binding cellular CBP (Swenson et al. 2001). KSHV Rta/Orf50 also binds HDAC1, a cellular histone deacetylase, and this binding decreases the ability of Rta/Orf50 to transactivate viral promoters (Gwack et al. 2001a). In addition, Sp1 binding sites are involved in Rta/Orf50 transactivation of many viral promoters (see Table 1 for references). Sp1 itself binds to the CBP/p300 coactivator complex, and the activity of Sp1 is repressed by HDAC1 in the absence of viral infection (Doetzlhofer et al. 1999), suggesting a complex interplay among these important modifiers of basal transcription.

The development and characterization of KSHV Rta/Orf50 DNA-binding mutants, which display either enhanced or abolished DNA binding to RREs, has enabled classifications of Rta/Orf50-responsive promoters into either of two subgroups: those where Rta/Orf50 directly binds promoter DNA and those where Rta/Orf50 does not directly bind promoter DNA but rather transactivates by protein-protein interactions with cellular transcription factors, including RBP-J- κ (Chang et al. 2005). These mutants will no doubt facilitate further clarification of Rta/Orf50-responsive promoters as to which mechanism Rta/Orf50 transactivates and which cellular pathways are involved.

9

Repression of Rta/Orf50 Transactivation

Rta/Orf50 interacts with the viral early protein K-bZip, encoded by the K8 open reading frame. This protein-protein interaction leads to repression of Rta's ability to transactivate *in vitro* (Liao et al. 2003a, 2003b). K-bZip is a homologue of EBV Zta, and accumulating evidence suggests a role for K-bZip in DNA replication and transactivation. K-bZip repressed Rta/Orf50 transactivation of the Orf57/Mta and K8/Kb-Zip promoters but had no effect

on Rta/Orf50's transactivation of the PAN/nut-1 promoter, demonstrating promoter-specific repression by K-bZip. The leucine zipper domain (aa 190–237) of K-bZip seems to be required for Rta/Orf50 binding (Liao et al. 2003b).

In addition to viral proteins, cellular interferon response factor 7 (IRF7) was reported to decrease transactivation of the Mta/Orf57 promoter by competing with Rta/Orf50 for binding to the RRE. Interferon- α was also shown to decrease transactivation of the Mta/Orf57 promoter by Rta/Orf50, with this process still involving IRF7 (Wang et al. 2005). This is consistent with the observation that interferon- α inhibits KSHV reactivation in PEL (Chang et al. 2000; Zoetewij et al. 1999; Pozharskaya et al. 2004). In contrast, Hayward and colleagues have reported an E3 ubiquitin ligase activity of KSHV Rta/Orf50 and that Rta directs polyubiquitination of cellular IRF7 leading to proteosomal degradation of IRF7 and blockage of IRF7-mediated expression of type I interferon transcripts (Yu et al. 2005). Because Rta/Orf50 is the principal regulator of KSHV reactivation it seems logical that this protein serves as the nexus between virus and antiviral response.

The chemical compound methotrexate was shown to downregulate KSHV Rta/Orf50-mediated transactivation and inhibit lytic reactivation (Curreli et al. 2002). This is consistent with the previously reported antiviral activity of methotrexate for other herpesviruses (Lembo et al. 1999; Shanley and Debs 1989). Yet there are also reports that methotrexate induces EBV reactivation (Feng et al. 2004). Most likely the systemic antiviral activity of methotrexate is related to its action as an antimetabolite and dihydrofolate reductase inhibitor, while its effects on Rta/Orf50 transactivation may be a by-product of cellular stress signaling induced by imbalances in intracellular nucleotide pools.

Interestingly, the KSHV latency-associated nuclear antigen (LANA) protein is capable of repressing transcription of the Rta/Orf50 promoter (Lan et al. 2004). This repression is dependent on the presence of RBP-J- κ -responsive elements found within the Orf50 promoter (Lan et al. 2005). Lan et al. also showed that LANA bound Rta/Orf50 protein directly (Lan et al. 2005). RRV LANA (R-LANA), like KSHV LANA, represses the transactivation ability of RRV Rta/Orf50, and this repression is reversed by treatment with the histone deacetylase inhibitor TSA (DeWire and Damania 2005). The HVS C488 LANA also suppresses the HVS Rta/Orf50 (Schafer et al. 2003), which fits a model in which LANA, the latent transactivator, and Rta/Orf50, the lytic transactivator, counterbalance each other. This balance of power is evolutionarily conserved among the rhadinoviruses, although the governing molecular mechanisms may be different. The outcome of the Rta/Orf50–LANA/Orf73 power struggle eventually determines whether the virus persists latently or reactivates, which in turn results in a profound difference in overall viral persistence and pathogenesis within the infected host.

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Structure and Function of Latency-Associated Nuclear Antigen

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1	Introduction	102
2	Expression of LANA	103
2.1	Tissue-Specific Expression of LANA	103
2.2	Levels of LANA mRNA in Infected Cells	105
3	Structure and Characterization of LANA	107
4	The Multifunctional Role of LANA	110
4.1	LANA as a Transcriptional Modulator	110
4.1.1	LANA Contributes to Maintenance of Viral Latency by Regulating RTA Function	111
4.1.1.1	LANA Represses RTA Expression	111
4.1.1.2	RTA Upregulates LANA After De Novo Infection	112
4.2	Deregulation of Cellular Factors by LANA	113
4.2.1	Role of LANA on Action of GSK-3 β and β -Catenin in Cell Cycle Deregulation	113
4.2.2	LANA Regulates p53 Activity	115
4.2.3	LANA Inhibits Rb-Induced Growth Arrest	116
4.2.4	LANA Associates with RING3/Brd2	117
4.2.5	The Interferon-Inducible Protein MNDA Binds to LANA	118
4.2.6	LANA Associates with the Acetyl Transferase pCBP and p300	119
4.2.7	LANA Induces Expression of the Id Proteins Involved in Regulating Cell Differentiation	120
4.3	Role of LANA in Episome Maintenance	121
4.4	Viral DNA Replication	123
5	Overview of LANA's Role in KSHV Pathogenesis	124
	References	125

Abstract Latency-associated nuclear antigen (LANA) encoded by open reading frame 73 (ORF73) is the major latent protein expressed in all forms of KSHV-associated malignancies. LANA is a large (222–234 kDa) nuclear protein that interacts with various

cellular as well as viral proteins. LANA has been classified as an oncogenic protein as it dysregulates various cellular pathways including tumor suppressor pathways associated with pRb and p53 and can transform primary rat embryo fibroblasts in cooperation with the cellular oncogene *Hras*. It associates with GSK-3 β , an important modulator of Wnt signaling pathway leading to the accumulation of cytoplasmic β -catenin, which upregulates Tcf/Lef regulated genes after entering into the nucleus. LANA also blocks the expression of RTA, the reactivation transcriptional activator, which is critical for the latency to lytic switch, and thus helps in maintaining viral latency. LANA tethers the viral episomal DNA to the host chromosomes by directly binding to its cognate binding sequence within the TR region of the genome through its C terminus and to the nucleosomes through the N terminus of the molecule. Tethering to the host chromosomes helps in efficient partitioning of the viral episomes in the dividing cells. Disruptions of LANA expression led to reduction in the episomal copies of the viral DNA, supporting its role in persistence of the viral DNA. The functions known so far suggest that LANA is a key player in KSHV-mediated pathogenesis.

1

Introduction

The search for an etiological agent associated with Kaposi sarcoma (KS) lesions led to the identification of Kaposi sarcoma-associated herpesvirus (KSHV) as a likely candidate as biological cofactor in KS development (Chang et al. 1994). KS lesions comprise layers of spindle cells expressing lymphatic endothelial, smooth muscle, macrophage, and/or dendritic cell marker (Weninger et al. 1999). During the onset of KS, only 10% of the spindle cells are KSHV positive, but they progress to approximately 90% during the late stage of KS lesions (Boshoff et al. 1995; Staskus et al. 1997; Dupin et al. 1999; Sturzl et al. 1999). These infected cells express the latency-associated nuclear antigen (LANA) (Rainbow et al. 1997; Sarid et al. 1999).

The KSHV genome comprises an approximately 140-Kbp-long unique coding region that is flanked by multiple terminal repeats of 801 bp with high GC content (Russo et al. 1996). It is believed that the long unique region encodes for approximately 90 open reading frames (ORFs), although it is not known whether each of these encodes for a functional gene product (Russo et al. 1996). On infection, KSHV enters a quiescent period known as latent infection whereby the viral genome circularizes and exists as nuclear episomes through multiple host cell divisions (Renne et al. 1996). During this period, the virus limits gene expression to a specific subset of viral proteins, presumably to minimize the elicited host immune response (Zhong et al. 1996). This subset of protein includes LANA encoded by ORF73 (Kedes et al. 1997; Kellam et al. 1997; Rainbow et al. 1997), viral cyclin encoded by ORF72, and viral Fas-

associated death domain (FAAD) interleukin-1 β -converting enzyme (FLICE) inhibitory protein (*v*-FLIP) encoded by ORF71. These adjacent genes are cotranscribed on two polycistronic mRNAs, LT1 (LANA, *v*-cyclin, *v*-FLIP) or LT 2 (*v*-cyclin, *v*-FLIP) (Dittmer et al. 1998; Sarid et al. 1999; Talbot et al. 1999).

Analysis of the infected cells for latent viral infection by immunofluorescence and immunohistochemistry indicated that the LANA protein is present in almost all KSHV-infected cells of KS, primary effusion lymphoma (PEL), and multicentric Castleman disease (MCD) (Rainbow et al. 1997; Dupin et al. 1999; Katano et al. 2000; Parravicini et al. 2000). However, *v*-cyclin and *v*-FLIP have been shown to be predominantly expressed in PEL cell lines (Platt et al. 2000; Low et al. 2001). As the majority of the tumor cells are latently infected (Boshoff et al. 1995; Staskus et al. 1997; Dittmer et al. 1998; Chang et al. 2000), latent, not lytic, antigens are believed to be involved in tumorigenesis, although additional studies have shown that some lytic antigens including K1, GPCR, and ORF37, the DNA exonuclease involved in RNA degradation and initiation of host cell gene expression (Lee et al. 1998; Couty et al. 2001; Jeong et al. 2001; Nador et al. 2001; Glaunsinger and Ganem 2004), are important mediators of KSHV-induced pathogenesis. Thus the viral components expressed during this phase of the viral life cycle are most likely the players for disease manifestation (Schulz 1999). This places attention on LANA as a major effector of KSHV pathogenesis.

2

Expression of LANA

2.1

Tissue-Specific Expression of LANA

KSHV becomes latent after infection in the majority of the cells it infects. During this quiescent stage of the viral life cycle, the virus exists in the nucleus as an episomal DNA (Renne et al. 1996). The viral DNA is typically copied by the host DNA replication machinery during cell division. During mitosis, KSHV DNA is tethered through specific sequences on the TR and to histone H1 on host chromatin via the KSHV-encoded LANA. Therefore, LANA is thought to play a central role in maintaining viral latency (Ballestas et al. 1999b; Cotter and Robertson 1999; Ballestas and Kaye 2001).

In more than 90% of infected tumor cells, KSHV is locked into latent infection, which is likely to be stringently controlled. Protein expression is restricted to only a few viral genes. Most prominent among these is LANA, which is involved in episome maintenance and transcriptional regulation

associated with viral oncogenesis (Zhong et al. 1996; Kedes et al. 1997; Kellam et al. 1997; Rainbow et al. 1997). LANA mRNA transcription is shown to be under the control of LANA promoter (LANAp) (Sarid et al. 1998; Jeong et al. 2001; Renne et al. 2001). LANAp is active in the absence of other viral gene products in transient assays and in latent PEL and KS tumor cells (Jeong et al. 2001, 2004; Renne et al. 2001). ORF73 (encoding LANA), ORF72 (v-cyclinD), and ORF71 (vFLICE) are all expressed from the same locus in polycistronic, differentially spliced mRNAs whose transcription is coordinately regulated by a common promoter; these three ORFs form a major latent cassette (Cesarman et al. 1996; Dittmer et al. 1998; Sarid et al. 1999; Talbot et al. 1999; Grundhoff and Ganem 2001). Moreover, the LANAp is bidirectional, controlling the constitutive expression of the latent genes to the left but lytic, TPA-inducible expression of the K14 and ORF74/vGPCR to the right (Fig. 1). Interestingly, LANA can autoactivate its own promoter during latent infection, so ensuring that the levels of LANA are adequate for maintenance of latency (Jeong et al. 2001, 2004). Recently, Pearce et al. and Samols et al. demonstrated the encoding of microRNAs from the latency transcriptional units, ORF71, -72, and -73 (Pearce et al. 2005; Samols et al. 2005). microRNAs are noncoding regulatory RNA molecules that bind to 3'-untranslated regions (UTRs) of mRNAs to either prevent their translation or induce their degradation, thereby regulating gene expression (Ambros 2004; Bartel 2004).

Generally, KSHV persists in KS and PEL tumor cells, which are of endothelial and B-cell origin, respectively. In asymptomatic carriers, KSHV is found in CD19-positive peripheral blood mononuclear cells (Mesri et al. 1996;

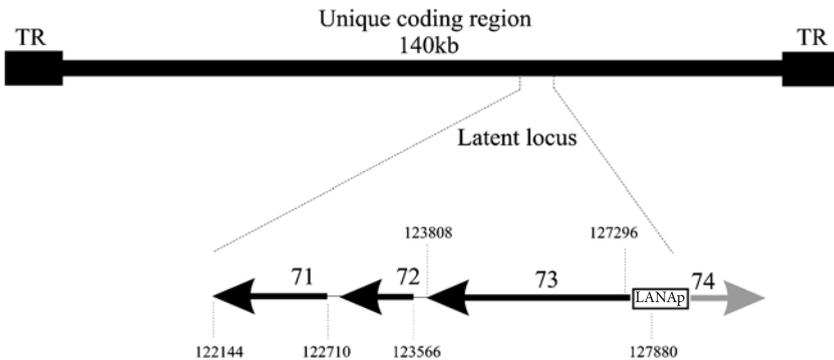


Fig. 1 Schematic diagram of the major KSHV latency cassette. Location of the major latency transcripts cluster in KSHV genome is shown. Numbers indicate nucleotide positions, based on the database sequence NC_003409 (Russo et al. 1996). *Black boldface arrows* represent ORF71, 72, and 73; *gray boldface arrow* represents ORF74

Jeong et al. 2002). However, KSHV can infect various human cell lines such as primary fibroblasts, 293 cells, and human primary vascular endothelial cell preparations with low efficiency as well as animal cells such as baby hamster kidney (BHK) cells in tissue culture conditions (Renne et al. 1998; Lagunoff et al. 2002; Bechtel et al. 2003). The KSHV LANA protein is predominantly expressed in the context of primary infection, whereas lytic genes are infrequently transcribed (Sun et al. 1999; Paulose-Murphy et al. 2001). Similarly, LANAp is active in a wide range of cell lines after transient transfection in culture (Jeong et al. 2001, 2004). However, in a SCID-hu Thy/Liv mouse model of de novo KSHV infection, LANA mRNA was transcribed in CD19-positive B cells but not in the more abundant T-cell subsets (Dittmer et al. 1999). Dittmer's group also found that a region extending to -1299 bp upstream of the LANA transcription start site was able to drive *lac Z*-reporter gene expression in several lines of transgenic mice. In agreement with KSHV's natural tropism, reporter gene expression was detected in CD19-positive B cells but not in CD3-positive T cells. Surprisingly, reporter gene expression was also detected in the kidney and in the liver at a lower level. In contrast to KS tumors, transgene expression was localized to kidney tubular epithelium rather than vascular endothelial cells (Jeong et al. 2002). This suggests that this particular promoter fragment contains all *cis*-regulatory elements sufficient for B-cell specificity but is likely to lack those required for endothelial specificity. These *in vivo* studies reflect a conundrum in KSHV biology: In culture, KSHV is able to infect a variety of cell types indiscriminantly, whereas in healthy latent carriers KSHV is found in B lymphocytes (Lagunoff et al. 2002). The transgenic mouse experiments suggest that there are tissue-restricted mechanisms regulating LANA gene expression, providing one possible explanation for B cell-specific viral persistence (Jeong et al. 2002).

2.2

Levels of LANA mRNA in Infected Cells

Like other herpesvirus, KSHV also displays a tightly controlled program of gene expression. KSHV can enter one of two modes of infection: (a) latent infection, in which the viral genome persists in its host cell with restricted gene expression without cell destruction (Zhong et al. 1996), and (b) lytic infection, which produces virion progeny and destroys the host cell (Sarid et al. 1998). Lytic infection, in turn, can be divided into immediate-early, early, and late stages. With KSHV DNA fragments as a probe, three classes of differentially transcribed messages were defined in a KSHV-infected PEL cell line (Sarid et al. 1998). Class I mRNAs can be detected in untreated cultures and are not increased after 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment,

which reactivates the KSHV lytic replication cycle. Class II mRNAs can be detected in untreated cultures but are greatly increased by TPA treatment. Class III mRNAs can only be detected in TPA-treated cultures (Sarid et al. 1998). Translated into the customary herpesvirus classification, class III mRNAs correspond to lytic transcripts and class I mRNAs correspond to latent transcripts. Class II mRNAs appear to be a unique set of transcripts, because it is not clear whether the mRNAs detected in untreated cultures stem from the few percent of cells that undergo spontaneous lytic reactivation in culture. KSHV-specific cDNA arrays have also confirmed and expanded these data (Jenner et al. 2001; Paulose-Murphy et al. 2001). Although KSHV lytic transcription appears to be an all-or-nothing response, which proceeds to completion once *orf50* (RTA) is expressed, KSHV can enter very distinct latency programs in terms of temporal regulation (latency type one or two) and tissue specificity (KS endothelial vs. B cell lineage). The type one latency mRNAs are resistant to the effects of the known chemical inducers such as ionomycin or butyrate, as well as TPA (Fakhari and Dittmer 2002). This suggests that LANAp, the promoter for LANA, v-FLIP, and v-cyclin, and the LANA-2/v-IRF-3 promoter share common regulatory features. The fact that latency type one mRNAs do not increase on viral reactivation in BCBL-1 cells suggests that their expression might be latency specific and that latency type one mRNAs might be expressed in latently infected cells to the exclusion of lytic mRNAs. Alternatively, latency type one mRNAs might be constitutively transcribed, but their promoters are unaffected or protected from the *orf50*-mediated upregulation of neighboring lytic transcription units (Fakhari and Dittmer 2002).

A number of studies showed that the mRNA level for LANA is unchanged after viral reactivation induced by TPA (Dittmer et al. 1998; Dittmer 2003). However, Jung and colleagues demonstrated opposite results by using a RTA tetracycline-inducible BCBL1 cell line (Nakamura et al. 2003). Under induced conditions, RTA expression is turned on, and in this system they were able to monitor changes in all viral transcripts. Interestingly, LANA mRNA levels are dramatically upregulated (Nakamura et al. 2003). RTA was shown to play a critical role in KSHV lytic reactivation, and forced expression of RTA was shown to initiate the full program of lytic replication (Lukac et al. 1998, 1999). These results suggest that LANA expression level may be changed during the natural reactivation process. In support of these studies, Chandran and colleagues showed that LANA transcripts were turned on by 48 h after infection (Krishnan et al. 2004).

3 Structure and Characterization of LANA

Sera obtained from HHV-8-infected patients were shown to recognize a specific nuclear antigen in latently infected PEL cell lines or KS tissue. This was characterized by a punctate nuclear immunofluorescence pattern (Lennette et al. 1996) (Fig. 2). Screening of cDNA libraries with serum from a HHV-8-positive patient identified this nuclear antigen as the product of the viral gene open reading frame 73 (ORF73), and the encoded protein was designated LANA (Kedes et al. 1997; Kellam et al. 1997; Rainbow et al. 1997). LANA is expressed from a latently controlled 5.32-kb transcript that also encodes the viral cyclin (v-Cyc) and v-FLIP (Dittmer et al. 1998). The 5.32-kb latent transcript is spliced to form a 1.7-kb transcript that encodes only v-Cyc and v-FLIP (Kellam et al. 1997; Dittmer et al. 1998). The predicted LANA protein is 1,162 amino acids (aa) and has a theoretical molecular mass of 135 kDa. In contrast to LANA's predicted molecular size, this protein has a much higher molecular size of 220–230 kDa when analyzed by Western blotting (Gao et al. 1996; Kellam et al. 1997; Rainbow et al. 1997). LANA is divided into three distinct domains: an N-terminal 337-amino acid domain; an extremely hydrophilic central domain of 585 aa consisting of multiple repeat elements predominantly containing the charged polar amino acids glutamine, glutamic acid, and asparagine; and a C-terminal 240-amino acid domain (Russo et al. 1996). In contrast to the central domain, the N- and C-terminal domains are rich in basic amino acids (Fig. 3). The differently charged domains of the protein may in part explain the aberrant running of LANA on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

To date, a number of studies have investigated the structural and functional aspects of LANA. In strain BC-1, LANA contains 1,162 aa (Russo et al. 1996) and has an apparent molecular mass of 224–234 kDa on SDS polyacrylamide gels (Rainbow et al. 1997). However, its size varies greatly among HHV-8

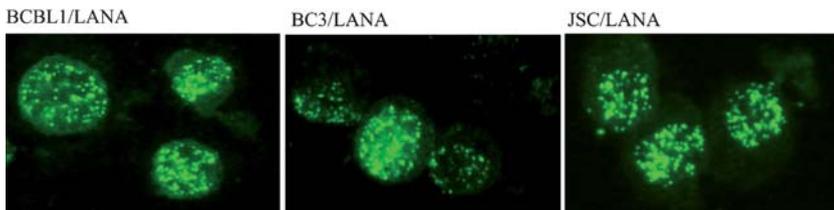


Fig. 2 LANA shows punctuate nuclear staining. Immunofluorescence analysis showed that LANA was localized to nucleus of the different KSHV latently infected B lymphoma cell lines BCBL1, BC3, and JSC-1

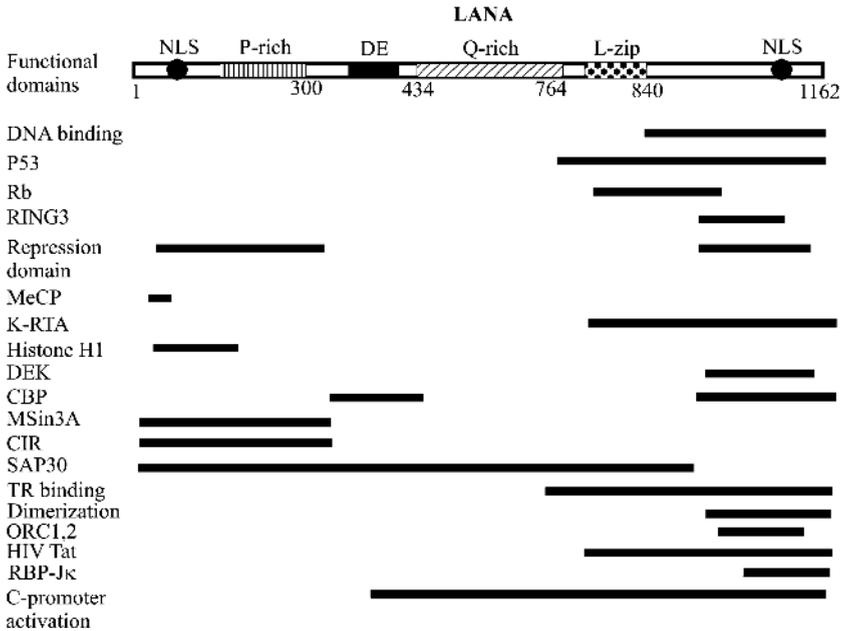


Fig. 3 Schematic showing the structure and functional domains of LANA. LANA is a 1162-amino acid protein nuclear protein (BC-1 strain). Numbers indicate the amino acids (*aa*). The putative domains include an N-terminal proline-rich domain (*P-rich*); aspartic acid, glutamic acid repeat region (*DE*); glutamine-rich domain (*Q-rich*); leucine zipper (*LZ*); LANA also has two nuclear localization signals (*NLS*) in both the N- and C-terminal regions. The approximate position of each functional domain is shown by a *black bar*, corresponding to the function shown in the column on the left (Cotter and Robertson 1999; Friberg et al. 1999; Platt et al. 1999; Krithivas et al. 2000; Schwam et al. 2000; Cotter et al. 2001; Groves et al. 2001; Hyun et al. 2001; Lim et al. 2001; Krithivas et al. 2002; Mattsson et al. 2002; Shinohara et al. 2002; Borah et al. 2004; Lan et al. 2004, 2005a)

isolates, mainly because of variations in length that affect the large internal acidic domain (Neipel et al. 1997; Rainbow et al. 1997; Gao et al. 1999; Glenn et al. 1999; Zhang et al. 2000). Sequence alignments of LANA from BC1, BBG1, KS1, and KS2 cell lines showed that LANA can be divided into two conserved regions, spanning aa 1 to 337 (the unique left region) and 798 to 1036 (the unique right region), that are separated by an internal region composed mainly of a limited number of repeated motifs (Piolot et al. 2001). Nucleotide alignments revealed that only four nucleotide positions are variable in the unique left region, whereas the unique right region was completely conserved

(Piolot et al. 2001). In contrast, high variability was observed in the central acidic domain, at both the nucleotide and amino acid levels. The region encoding aa 338–797 is composed of a series of short conserved tandem repeats whose number is characteristic of each HHV-8 isolate. This region is interrupted by a conserved nonrepeated sequence (the unique central region) encompassing aa 439–452 (Piolot et al. 2001).

Studies have shown that LANA is localized in the nuclei of KSHV latently infected cells and that it interacts with the border of heterochromatin during interphase and with chromosomes during mitosis (Szekely et al. 1998, 1999; Ballestas et al. 1999b; Cotter and Robertson 1999). A unique nuclear localization signal (NLS) sequence was mapped within aa 24–30 of LANA (Szekely et al. 1999; Schwam et al. 2000; Piolot et al. 2001). The LANA NLS is highly homologous to the NLSs that have been identified in EBV EBNA1 (Schwam et al. 2000). In particular, these sequences have a phosphorylation site for protein kinase A and a cdc2-type kinase in common. Because phosphorylation of residues close to or within the NLS has been shown to modify the nuclear import of numerous cellular proteins, this suggests that LANA nuclear transport may be regulated by a similar kinase(s), notably during the course of the cell cycle (Boulikas 1993). Except for the N terminus, which was shown to be localized to nucleus, Wilson and colleagues demonstrated that both the N and C termini of LANA can localize to the nucleus. They showed that the N terminus gives a diffuse pattern in interphase nuclei typical of many transcription factors; however the C terminus accumulates as discrete nuclear speckles reminiscent of the punctate pattern shown by full-length LANA (Schalling et al. 1995; Friberg et al. 1999; Glenn et al. 1999).

It has also been shown that LANA self-associates to form dimers in the absence of viral DNA or other viral gene products (Schwam et al. 2000). Self-association is mediated by the C terminus of LANA, and, interestingly, the sequences required for self-association of LANA are conserved. The amino acid sequence of the C terminus of KSHV LANA with the similar regions of LANA homologs encoded by four other rhadinoviruses, ateline herpesvirus-3 (Ensser et al. 1997), herpesvirus saimiri (Albrecht et al. 1992), macaque rhadinovirus 17577 (Searles et al. 1999), and murine herpesvirus 68 (Virgin et al. 1997), when compared showed that the greatest conservation of the C-terminal sequence corresponds to residues 934–1072 of KSHV LANA, with a more limited conservation between residues 885 and 922 (Russo et al. 1996). Clearly, the C terminus of LANA (residues 884–1089) is required for self-association and includes the majority of the residues shown to be conserved between members of the Rhadinovirus family (Schwam et al. 2000).

Like EBNA1 of EBV, LANA tethers the viral episomes to host chromosomes, ensuring that the viral genome is segregated to daughter cells on mitosis (Cot-

ter and Robertson 1999; Schwam et al. 2000). LANA interacts with the mitotic chromosomes in KSHV-infected cells. The major chromosome-binding site of LANA was mapped to the N terminus between aa 1 and 32 (Schwam et al. 2000). This region also contains the LANA NLS; therefore it is possible that nuclear targeting and binding to mitotic chromosomes were functionally related (Schwam et al. 2000).

4

The Multifunctional Role of LANA

4.1

LANA as a Transcriptional Modulator

Numerous studies from a number of laboratories have demonstrated that LANA can activate as well as repress transcription (Groves et al. 2001; Hyun et al. 2001; Jeong et al. 2001; Knight et al. 2001; Lim et al. 2001; Renne et al. 2001; An et al. 2002). LANA has also been shown to autoactivate transcription of the major latent promoter (Renne et al. 2001; Jeong et al. 2004), suggesting a role in maintenance of expression of these latent transcripts while the majority of the viral genome remains silent. Transcriptional activation by LANA is probably independent of direct DNA binding as no specific LANA binding sequences have been identified in the major promoter elements (Russo et al. 1996). LANA activation of transcription is directed by a wide array of simple, synthetic promoters containing binding sites for a range of cellular proteins including ATF, AP-1, CAAT, or Sp1, linked to a TATA box (Renne et al. 2001). Although the TATA box alone was shown to be activated by LANA in these studies (Renne et al. 2001), activation of the cellular IL-6 promoter by LANA required both a TATA box and an upstream AP-1 element (An et al. 2002). Additionally, activation of the HIV-1 LTR in BJAB cells requires both the TATA box and the core enhancer elements (NF-IL-6, Ets, NF- κ B, and Sp1 sites) (Hyun et al. 2001). However, LANA can activate the HIV-1 LTR independently of the core enhancer if the Tat protein is coexpressed; this effect is mediated by the C-terminal 400 aa of LANA, which includes the third repeat region (Hyun et al. 2001). Truncated LANA containing the internal repeat domain (IRD) plus the C terminus activates the transcription of an EBV latency promoter containing PU.1, ATF, and Sp1 elements (Groves et al. 2001). Mechanistically, LANA can modify the DNA binding activity of Sp1 to activate the transcription of the telomerase reverse transcriptase promoter (Knight et al. 2001). Furthermore, LANA was shown to interact with Sp1 directly as well as associated in KSHV-infected cells, suggesting an upregulation of hTERT by directly interacting with Sp1 at the transcription domains (Verma et al. 2004).

LANA can also contribute to broad repressive effects on transcription. In Cos cells, LANA represses the transcriptional activity of NF- κ B. Additionally, repression of the HIV-1 LTR suggested that the interaction of LANA with the cellular transcription apparatus is cell specific (Renne et al. 2001). LANA also represses transcription controlled by the ATF4/CREB2 protein, independently of ATF4 DNA binding. This effect is mediated by LANA aa 751–1162, which includes the third repeat region (Lim et al. 2001). Similarly, the first acidic repeat (aa 340–431) of LANA is one of two domains required to competitively bind the cellular cyclic AMP-responsive element binding protein (CREB) binding protein (CBP) and block its histone acetyltransferase activity, thus inhibiting the ability of CBP to coactivate transcription with c-fos (Lim et al. 2001). This mechanism reflects the general ability of LANA to repress transcription driven by other cellular and viral CBP-dependent transactivators, including the KSHV lytic switch protein ORF50/Rta (Gwack et al. 2001). To determine the role of LANA on cellular pathways, an overexpression system followed by a microarray analysis revealed the induction of a large number of genes in LANA-expressing cells including pRb and p53 signaling molecules (An et al. 2005).

4.1.1

LANA Contributes to Maintenance of Viral Latency by Regulating RTA Function

4.1.1.1

LANA Represses RTA Expression

Recently, studies in our laboratory showed that LANA is capable of maintaining viral latency through repression of the transcriptional activity of RTA (replication and transcription activator) promoter (Lan et al. 2004). RTA is encoded by ORF50 of KSHV and is an immediate-early protein. Exogenous expression of RTA from a heterologous promoter or induction of endogenous RTA expression with chemical inducers can initiate lytic reactivation of KSHV (Lukac et al. 1998, 1999). HHV-8 RTA is the key transcriptional regulator that controls the switch from viral latency to lytic replication and is sufficient to drive the viral lytic cascade to completion, resulting in the production of viral progeny (Lukac et al. 1998, 1999). The RTA protein is made as an IE gene product; it autoregulates its own expression, and activates the transcription of various viral and cellular genes (Deng et al. 2000). It was shown that LANA downmodulates Rta's promoter activity in transient reporter assays, thus repressing the Rta-mediated transactivation (Lan et al. 2004). This eventually resulted in a decrease in the production of KSHV progeny (Lan et al. 2004). LANA physically interacts with Rta in vitro and associates with RTA in KSHV-infected cells (Lan et al. 2004). These results demonstrated that LANA

is capable of inhibiting viral lytic replication by repressing transcription and antagonizing the function of RTA (Lan et al. 2004). Therefore LANA provides a critical role in controlling the switch between viral latency and lytic replication (Lan et al. 2004). Similarly, Schafer and colleagues in a recent report also indicated that the LANA homologue in HVS can repress the expression of ORF50 encoded by HVS (Schafer et al. 2003). Most recently, DeWire and Damania also showed that the LANA homologue of rhesus monkey rhadinovirus (RRV) can dramatically decrease the transactivation function of RRV Orf50 (RTA) and inhibit RRV lytic replication *in vitro* (DeWire and Damania 2005). Interestingly, a recent report showed that KSHV could undergo spontaneous lytic replication, and RTA transcription level was increased when LANA expression was knocked down with specific siRNA (Godfrey et al. 2005). These findings provide one explanation as to why KSHV is predominantly maintained in the latent state in infected cells as well as why after the primary lytic infection, KSHV rapidly establishes latent infection. Moreover, it may be a common and conserved mechanism whereby LANA inhibits lytic reactivation through downmodulation of the immediate-early transactivator, RTA expression. Further, it has been shown that LANA downregulates RTA's promoter activity through the RBP-J κ binding sites within the promoter (Lan et al. 2005a). RBP-J κ is a sequence-specific DNA-binding protein in the CSL/CBF-1 family, normally thought to function as a major repressor of transcription in response to activation of the Notch pathway signaling. Our results once again showed that viral proteins can regulate gene expression through direct targeting of important cellular signaling pathways. Interestingly, Liang and colleagues also demonstrated that RTA can functionally interact with RBP-J κ and mimic cellular Notch function to activate lytic gene expression (Liang et al. 2002; Liang and Ganem 2003).

4.1.1.2

RTA Upregulates LANA After De Novo Infection

Most recently, it has been shown that RTA is detected in association with KSHV viral particles (Bechtel et al. 2005; Lan et al. 2005a, 2005b). This raises the possibility that RTA may contribute to latency establishment by inducing LANA expression upon entry. Interestingly, it was shown that RTA activates the LANA promoter and induces LANA expression in transient transfection experiments (Lan et al. 2005b). Moreover, the transcription of RTA correlates with that of LANA during the early stages of *de novo* infection of KSHV and LANA transcription is responsive to induction of RTA expression (Lan et al. 2005b). Additionally, the RBP-J κ binding site within the LANA promoter is also shown to be critical for regulation by RTA known to be associated

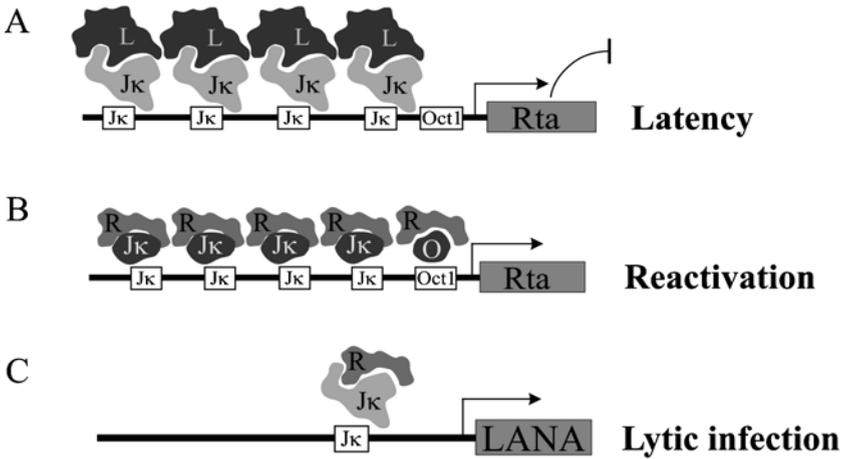


Fig. 4A–C Hypothetical model to show interplay between LANA and RTA. **A** During the latent infection of KSHV, LANA represses RTA expression through physical interaction with RBP-J κ . **B** On reactivation of the virus, RTA can autoactivate its own expression then initiate the cascade of lytic gene expression. **C** In early stage of primary infection, RTA can activate LANA expression through functional association with RBP-J κ

with a number of cellular regulatory proteins including RBP-J κ . In RBP-J κ -knockout cells KSHV was unable to establish latent infection efficiently compared to wild-type cells (Lan et al. 2005b). These findings therefore suggest that RTA can contribute to the establishment of latency through activation of LANA expression in the early stages of infection. This represents a feedback loop whereby LANA and Rta regulate each other and is likely to be, at least in part, a key event in establishment of KSHV latent infection. A potential model can be proposed that suggests a feedback regulatory loop between RTA and LANA and implies that the interplay between RTA and LANA could be the master control for the switch between lytic replication and latency of KSHV utilizing the major cellular Notch signaling pathway through targeting of the RBP-J κ effector protein (Fig. 4).

4.2

Deregulation of Cellular Factors by LANA

4.2.1

Role of LANA on Action of GSK-3 β and β -Catenin in Cell Cycle Deregulation

LANA, which is expressed in all forms of KS tumors, modulates distribution of glycogen synthase kinase (GSK)-3 β , a negative regulator of β -catenin

and Wnt signaling (Fujimuro et al. 2003). In normal cells cytosolic amounts of β -catenin are normally limiting because it leads to targeted degradation after phosphorylation with GSK-3 β (Polakis 2000). The nuclear effector protein β -catenin binds to the cytoplasmic domain of E-cadherin, which is the transmembrane protein present at adhesion junctions, and also to the cytoplasmic complex comprised of the serine-threonine kinase GSK-3 β , the tumor suppressor adenomatous polyposis coli (APC) and the scaffold protein Axin (Polakis 2000). Association of β -catenin with the above complex results in phosphorylation of β -catenin by GSK-3 β through association with the F-box protein β -TrCP leading to ubiquitination and thus proteosomal degradation by the specific E5 ligases (Kitagawa et al. 1999). Activation of Wnt signaling by binding of Wnt ligand to a frizzled (Fz)/low-density lipoprotein receptor-related protein (LRP) complex activates the cytoplasmic protein Disheveled (Dsh in *Drosophila* and Dvl in vertebrates), possibly through phosphorylation by casein kinase 1 (CK1) and casein kinase 2 (CK2) (Willert et al. 1997; Sakanaka et al. 1999). Dsh/Dvl then inhibits the activity of the multiprotein complex β -catenin-Axin-APC-GSK-3 β , which targets β -catenin for proteosomal degradation by phosphorylation, resulting in accumulation of cytosolic forms of β -catenin (Polakis 2000). Stabilized β -catenin translocates into the nucleus and binds to members of the T-cell factor (Tcf)/lymphoid enhancing factor (Lef) family of DNA binding proteins, leading to transcription of Wnt target genes (Boshoff 2003).

Fujimuro and Hayward reported increased levels of β -catenin in PEL cells (BC2, BC3, and BCBL1) (Fujimuro et al. 2003; Fujimuro and Hayward 2004). Immunohistochemical detection of β -catenin showed elevated expression of β -catenin in KS tumors compared to the surrounding normal tissues (Fujimuro et al. 2003). LANA, which is primarily expressed in all KSHV-infected cells, induced the accumulation of β -catenin (Fujimuro et al. 2003). The accumulation of β -catenin is possibly through interaction of LANA with GSK-3 β , which is important for phosphorylation of β -catenin and its degradation. LANA changes the distribution of GSK-3 β from predominantly cytoplasmic to nuclear, thus preventing phosphorylation of cytoplasmic β -catenin, which in turn translocates to the nucleus and forms a complex with the Tcf and Lef family of transcription factors to activate genes containing Tcf and Lef binding sites (He et al. 1998; Barker et al. 2000; Fujimuro and Hayward 2003, 2004; Fujimuro et al. 2003). These target genes include *MYC*, *JUN*, and *CCND1*, known to be critical in regulating cell proliferation (cyclin D1) (He et al. 1998; Boshoff 2003; Fujimuro et al. 2003) (Fig. 5).

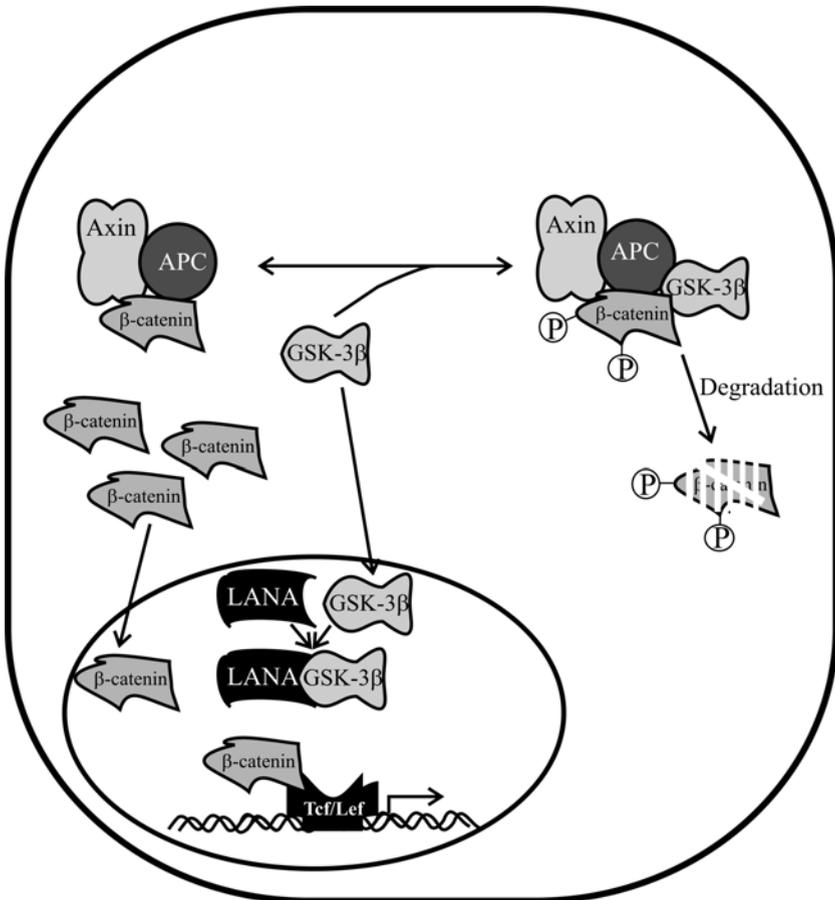


Fig. 5 Model showing modulation of Wnt signaling pathways through interaction with LANA. LANA binds to GSK-3 β and translocates this to the nucleus, leading to the depletion of cytoplasmic GSK-3 β . This results in accumulation of unphosphorylated β -catenin, which in turn translocates to the nucleus and associates with the LEF and TCF family of transcription factors, thus modulating gene expression controlled by these transcription factors

4.2.2

LANA Regulates p53 Activity

p53 was the first identified tumor suppressor gene, and the induction of this pathway eliminates or inhibits the proliferation of abnormal cells, thereby preventing neoplastic development (Hollstein et al. 1991; Friend 1994; Levine

1997). Mutations identified in the p53 gene are estimated to occur in 50% of all cancers, and loss of p53 function leads to cell transformation and oncogenesis (Hollstein et al. 1991; Levine 1997). Besides mutation in the gene, several viral oncoproteins have been shown to interact with p53 and modulate its transcriptional activity (Chen et al. 1996; Ko and Prives 1996). HPV-16-encoded E6 binds with ubiquitin ligase to mediate p53 ubiquitination and degradation (Werness et al. 1990). SV40 large T antigen modulates p53-mediated apoptosis by physically interacting with and inactivating p53 (Jay et al. 1981). More recently, Nabel and colleagues have shown that LANA interacts with p53 and downregulates its transcriptional activity as well as blocking its ability to induce apoptosis (Friborg et al. 1999). Modulation of p53 transcriptional activity by LANA is neither through degradation nor inhibition of its ability to bind DNA. This raises the possibility that additional factors are required for its function (Friborg et al. 1999). The region of LANA that binds to p53 was mapped to aa 441–1162, which is further narrowed down to aa 751–1162 (Borah et al. 2004) (Fig. 1). The herpesvirus positional homologue of LANA also binds to p53 and most probably performs a similar function (Borah et al. 2004). The domain of p53 that maps to bind KSHV-encoded LANA and its HVS ORF73 homologue is the tetramerization domain essential for enzymatic activity, but the presence of LANA did not affect the tetramerization of p53, suggesting that inactivation of p53 function by LANA may involve another potential mechanism besides that which involves tetramer disruption (Borah et al. 2004). Recently, Si and Robertson reported that LANA-expressing cells had increased chromosomal instability, detected by the presence of increased multinucleation, micronuclei, and aberrant centrosomes (Si and Robertson 2006). They proposed that repression of p53 functional activity contributes to the chromosomal instability, thereby facilitating KSHV-mediated pathogenesis (Si and Robertson 2006).

4.2.3

LANA Inhibits Rb-Induced Growth Arrest

Retinoblastoma (pRB) acts as a negative regulator of cell cycle progression by blocking the cells from entering into S phase from G₁ (Sherr and McCormick 2002). In early G₁, pRB is hypophosphorylated and associates with specific members of the E2F transcription factor, finally resulting in transcriptional repression (Kiess et al. 1995). Cell cycle progression from G₁ to S phase occurs when pRB is hyperphosphorylated by complexes of D-type cyclins/CDK4/CDK6, thereby releasing the E2F transcription factor. This results in activation of the DNA replication machinery and nucleotide synthesis to prepare the cell for division (Dyson 1998). LANA has been shown to bind and

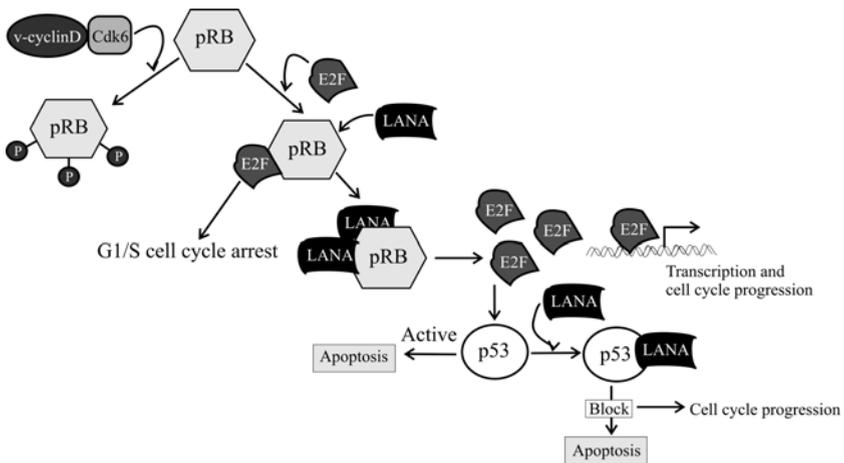


Fig. 6 Modulation of pRB and p53 pathways in KSHV-infected cells. Latency-associated nuclear antigen (LANA) competes with E2F transcription factor for binding to pRB and releases the E2F transcription, which leads to the upregulation of E2F-modulated genes. LANA also interacts with p53 and blocks the p53-mediated apoptosis, thus helping in cell cycle progression and tumorigenesis

inactivate pRb, thereby upregulating transcription of the promoter containing the cell cycle transcription factor E2F DNA-binding sequences (Radkov et al. 2000). Exogenous expression of LANA in SAOS-2 cells overcomes pRb-induced growth arrest and drives the cells into S phase (Radkov et al. 2000). LANA can transform primary rat embryo fibroblasts and make them tumorigenic in cooperation with the cellular oncogene Harvey rat sarcoma viral oncogene homologue (*Hras*) (Radkov et al. 2000). Additionally, the LANA homologue of herpesvirus saimiri ORF73 can also bind to pRb, resulting in upregulation of E2F responsive promoter activity (Borah et al. 2004) (Fig. 6).

4.2.4

LANA Associates with RING3/Brd2

RING3 is a ubiquitously expressed group of proteins in mammalian cells (Beck et al. 1992; Apone et al. 1996). They are bromodomain-containing proteins that include the SWI/SNF-type chromatin remodeling proteins that have elevated activity in human leukemia (Denis and Green 1996). LANA binds to RING3 through the extraterminal (ET) domain, characteristic of fsh-related proteins, suggesting a highly conserved function in terms of protein-protein interactions (Beck et al. 1992; Nomura et al. 1994; Jones et al. 1997). Proteins belonging to this group have been shown to be involved in cell cycle control.

Interaction of RING3/Brd2 shown with nuclear extract protein complex has been hypothesized to promote G₁-S transition (Denis et al. 2000; Guo et al. 2000). The interaction between LANA and RING3 leads to the phosphorylation of serine and threonine residues in the C terminus of LANA, between residues 951 and 1107 (Platt et al. 1999). This group of proteins also includes the yeast BDF proteins that regulate transcription through interaction with general transcription factors (Guo et al. 2000). RING3 localizes to euchromatin regions in the interphase nucleus even in the absence of KSHV viral genome and is released to the cytoplasm during mitosis. In KSHV-infected cells most of the RING3 proteins colocalized with LANA, suggesting that RING3 may contribute to KSHV genome persistence by local euchromatin microenvironment around the viral episomes tethered to the heterochromatin modulating transcription or promoter replication-segregation of the KSHV genome (Mattsson et al. 2002). Very recently RING3/Brd2 binding domain of LANA was mapped lying between aa 1007 and 1055 (Viejo-Borbolla et al. 2005). Mutants of LANA capable of supporting replication and dimerization were able to interact with RING3/Brd2, suggesting that RING3 may be important for interaction of C-terminal domain of LANA with heterochromatin and its functional activities (Viejo-Borbolla et al. 2005).

4.2.5

The Interferon-Inducible Protein MNDA Binds to LANA

MDNA is a nuclear protein expressed selectively in hematopoietic cells and has been shown to be induced by interferon (IFN) (Johnstone and Trapani 1999). This protein, like other proteins of this family, human IFI16, AIM2 and murine p202, p203, 204, and D3 protein (hematopoietic interferon-inducible nuclear protein), contains a conserved 200-aa region (Johnstone and Trapani 1999). LANA binds to MNDA through its amino-terminal region (aa 22–274) (Fukushi et al. 2003). This region of LANA has also been shown to be involved in transcriptional repression (Lim et al. 2000; Schwam et al. 2000). LANA colocalizes with MNDA in PEL cells and also is overexpressed in HEK cells (Fukushi et al. 2003). The primary function of IFN in infected cells is the inhibition of the growth of virus-infected cells, but exogenous expression of IFN did not affect growth of KSHV-infected cells (Fukushi et al. 2003). These studies suggest that MDNA alone is not enough for IFN-induced growth inhibition of KSHV-infected cells (Fukushi et al. 2003).

LANA has been shown to bind with CBP and interferes with the interaction of CBP with c-Fos, a representative C/H3 region binding cellular transcription factor, *in vivo* and *in vitro* (Lim et al. 2001). Additionally, LANA inhibits transcriptional activity and also the *in vitro* histone acetyltransferase activity of CBP, suggesting that LANA modulates the global transcriptional activities of infected cells at least in part through interaction with CBP (Lim et al. 2001).

4.2.7

LANA Induces Expression of the Id Proteins Involved in Regulating Cell Differentiation

Id proteins belong to a family of four helix-loop-helix (HLH) proteins (Id-1 to Id-4) that were initially recognized as growth factor-inducible genes that inhibited cell differentiation (Benezra et al. 1990). These proteins are also referred to as inhibitors of DNA binding (Id) because they lack the basic amino acid sequence characteristic of basic bHLH transcription factors necessary for DNA binding. They also form heterodimers with transcription factors, inhibiting their ability to bind DNA (Benezra et al. 1990; Norton 2000). Recently various biological roles have been assigned to the Id family of proteins, including cell cycle regulation, embryonic development, cell death, and tumorigenesis (Nickoloff et al. 2000). These recently described functions may be through interaction with non-HLH proteins including the pocket domain proteins, pRb, p107 and p130, MIDA1, and ETS transcription factors (Hollnagel et al. 1999; Yates et al. 1999). Id proteins also promote cell progression through dysregulation of expression of p21, inhibitor of the cell cycle-dependent kinase (CDKI). Moreover, Id-1 regulates cell proliferation, possibly through direct interactions with the p16 promoter and/or inactivation of ETS2 transcription factors (Alani et al. 2001). Expression profile of Id-1 in human tumors, including squamous cell carcinoma and endometrial, breast, and cervical cancers, showed a significant level of expression compared to normal cells (Langlands et al. 2000; Schindl et al. 2001; Tang et al. 2003). Higher levels of expression have also been found in invasive or high-grade tumors, indicating a possible correlation with the more aggressive form of the disease (Lin et al. 2000). Overexpression of Id-1 in keratinocytes and endothelial cells (ECs) has been shown to delay onset of cellular senescence (Tang et al. 2003). KS tumors showed elevated levels of Id-1 expression, and infection with KSHV drastically increased Id-1 expression (Tang et al. 2003). Ectopic expression of LANA in endothelial cells increased Id-1 expression, suggesting that LANA may drive cell proliferation by inducing Id-1 expression (Tang et al. 2003).

4.3

Role of LANA in Episome Maintenance

Persistence of KSHV through successive rounds of cell division in new daughter cells is enhanced by the partitioning of the viral DNA to host daughter cells during mitosis to prevent loss of the viral DNA after the cell divides. Studies have demonstrated that LANA associates with the KSHV genome in infected cells and colocalizes with the viral genome both in interphase nuclei and on mitotic chromatin in a punctate pattern (Ballestas et al. 1999b; Cotter and Robertson 1999). The ability of LANA to support persistence of the KSHV genome is crucial for the establishment of latency. LANA has been shown to target heterochromatin-associated nuclear bodies and selectively associates with human chromatin in human mouse hybrids containing a single fused nucleus (Szekely et al. 1999). The presence of LANA was shown to be essential for the long-term maintenance of the KSHV-derived Z6 cosmids (Ballestas et al. 1999b; Cotter and Robertson 1999). The above reports showed colocalization of LANA with the viral genome in the nucleus, demonstrated its role in episomal persistence, and additionally suggested its role in partitioning of the viral genome during mitosis. Recombinant KSHV virus cloned in a bacterial artificial chromosome (BAC36 Δ LANA) disrupted for LANA expression did not maintain viral episome, and the cells became virus free after 2 weeks of selection, confirming LANA's role in viral episome maintenance (Ye et al. 2004). Recently, Godfrey and colleagues used a short hairpin RNA (shRNA) approach to knock down the expression of the oncogenic latent gene cluster including LANA (Godfrey et al. 2005). Independent silencing of LANA did not completely knock down expression of LANA or v-cyclin but reduced it to 20% compared to control treatments. The reduction in the expression of LANA gradually reduced KSHV copy number until it achieved a lower but steady copy number (Godfrey et al. 2005). Indirect binding studies with KSHV DNA fragments indicated that LANA displays preferential binding to different regions of the KSHV genome, with at least three regions showing relatively stronger binding compared to the entire genome (Cotter and Robertson 1999). A small 2-Kbp region at the left end was shown to have strongest binding to LANA, and the binding sequence was narrowed down to a 13-bp cognate sequence in the TR region of the KSHV genome (Ballestas and Kaye 2001; Cotter et al. 2001; Renne et al. 2001). The binding region of LANA was initially mapped to the last 200 aa of the carboxy terminus and is now further mapped to residues 996–1139 (Komatsu et al. 2004). Scanning deletions within this region ablated both LANA oligomerization and DNA binding, consistent with a requirement for oligomerization to bind DNA (Komatsu et al. 2004; Srinivasan et al. 2004). A region of the KSHV genome at

the left end as well as a single and three copies of the TR elements persisted in cells stably expressing LANA *in trans*, suggesting that this region in fact contained the *cis*-acting DNA element (Cotter and Robertson 1999; Ballestas and Kaye 2001; Renne et al. 2001). However, this does not exclude other regions of the genome as alternate elements capable of functioning as origins of replication during persistent infection. Interestingly, LANA showed affinity to two other regions of the KSHV genome, albeit to a lesser degree than the left end (Cotter and Robertson 1999). LANA was also shown to bind to histone H1 but not core histones and to tether the viral episomes to the host chromatin (Cotter and Robertson 1999). A deletion in the chromosome binding domain

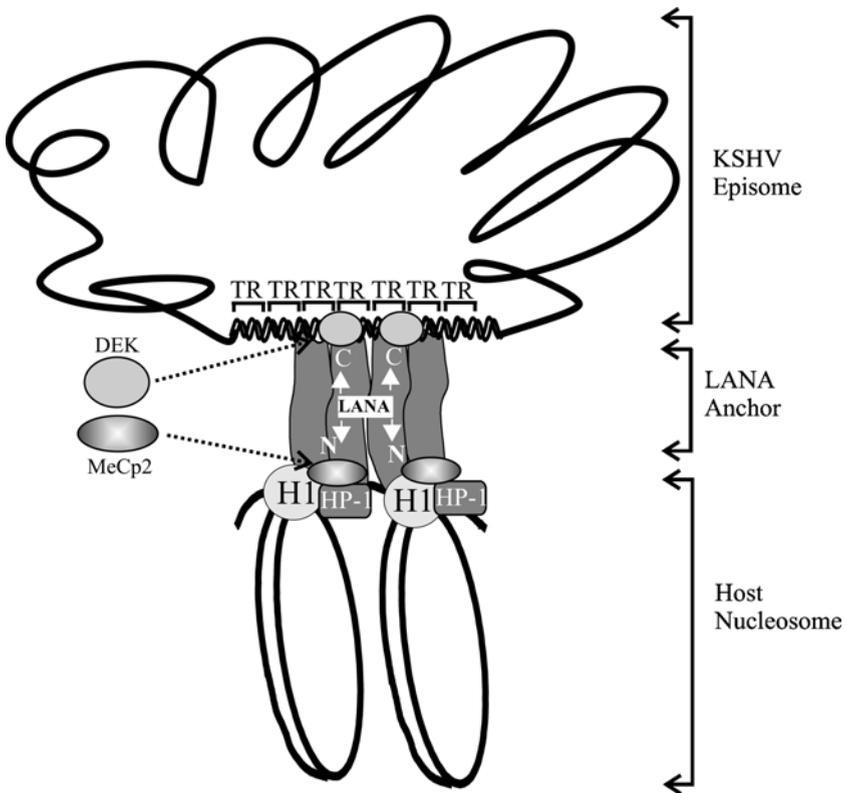


Fig. 8 Model of KSHV episomes tethering in KSHV-infected cells. C terminus of LANA binds to its cognate sequence in TR region of KSHV genome. N terminus of LANA attaches it to nucleosome with the help of cellular proteins including histone H1, heterochromatin protein 1 (HP-1). Cellular proteins MeCp2 and DEK help in tethering of the viral genome to the host chromosome

(CBD), aa 5–22 of the N-terminal region of LANA, abolished episomal maintenance but was restored by replacing the mutation with histone H1 protein (Shinohara et al. 2002). LANA therefore simultaneously associates with mitotic chromosomes via the CBD of its N terminus and through binding of its C terminus to the KSHV TR (Ballestas et al. 1999a; Piolot et al. 2001). These domains mediate chromosomal tethering, which may involve interactions with cellular proteins including the methyl CpG binding protein (MeCP2), heterochromatin proteins (HP-1) at N terminus, and DEK at C terminus of LANA protein (Krithivas et al. 2002) (Fig. 8). These studies demonstrate that LANA can function similar to EBNA1 in maintenance of the EBV episome in the infected cell (Cotter and Robertson 1999).

4.4

Viral DNA Replication

Replication of viral DNA is required for efficient segregation of viral episomes. LANA has been shown to be essential for the replication of the viral cosmid clone along with tethering to the host chromosomes (Ballestas et al. 1999b; Cotter and Robertson 1999). The TR elements of the KSHV genome have been proposed to be the potential site of initiation of replication by various groups using short-term replication assays (Hu et al. 2002; Lim et al. 2002; Grundhoff and Ganem 2003; Barbera et al. 2004; Komatsu et al. 2004; Hu and Renne 2005), although the replicative potential of other regions has not been thoroughly evaluated. Plasmids containing TR elements have been shown to replicate in LANA-expressing cells (Hu et al. 2002; Lim et al. 2002; Grundhoff and Ganem 2003; Hu and Renne 2005). Renne and colleagues performed deletion mapping of the TR region to identify minimal replicator sequences, and it was shown that a 71-bp-long minimal replicator region contained two distinctive sequence elements, LANA binding sites (LBS1/2). Additionally, adjacent 29- to 32-bp-long GC-rich sequences were essential for replication of the TR elements and thus could be termed a replication element (Hu and Renne 2005). In terms of proteins required for the replication of the viral episome, a number of cellular proteins have been proposed to be involved in this process. Human origin recognition complexes (hsORCs) have been shown to interact with LANA in *in vitro* experiments, most probably similar to EBV encoded EBV, which is believed to recruit cellular replication machinery at the Ori site (Dhar et al. 2001; Schneider 2001; Ritzi et al. 2003). The detailed mechanism of replication in KSHV is not well understood. However, recently it was shown that KSHV forms chromatin structures and the latent replication origin within the TR is bound by the LANA-associated proteins CBP, double-bromodomain-containing protein 2 (BRD2), and origin recognition

complex 2 protein (ORC2) (Stedman et al. 2004). This region is enriched in hyperacetylated histones H3 and H4 relative to other regions of the latent genome (Stedman et al. 2004). In slight contrast to previous studies, chromatin immunoprecipitation assays performed in our lab have shown that the association of cellular replication machinery proteins ORC2 and MCM3 occurs at various regions along the KSHV genome, suggesting that additional regions of the KSHV genome may be capable of functioning as origins of replication (Verma 2006).

5

Overview of LANA's Role in KSHV Pathogenesis

Numerous studies over the last 10 years have shown that LANA is a multi-functional protein ubiquitously expressed in KSHV-infected cells (Ballestas et al. 1999b; Cotter and Robertson 1999; Friberg et al. 1999; Radkov et al. 2000; An et al. 2002; Boshoff 2003; Cannon et al. 2003; Fujimuro et al. 2003; Fukushi et al. 2003; Verma and Robertson 2003; Borah et al. 2004; Cloutier et al. 2004; Lan et al. 2004; An et al. 2005). LANA modulates various cellular pathways both positively and negatively to drive cell proliferation. LANA also downregulates tumor suppressor pathways regulated by p53 and pRB as discussed earlier in this chapter. Interestingly, one study showed that KSHV can transform human umbilical vein endothelial cells (HUVEC) and that the levels of telomerase in these transformed cells were elevated (Flore et al. 1998). Telomerase is a holoenzyme that prevents chromosome degradation, end-to-end chromosome fusions, and chromosome loss and maintains telomere length by the addition of new repeat sequences at the ends of the chromosomes, thus deriving cell immortalization (Greider 1991a, b; Harrington and Greider 1991). In support of these studies LANA has also been shown to upregulate the human telomerase promoter through interaction with Sp1 transcription factor, supporting the role of LANA as an oncoprotein (Knight et al. 2001; Verma et al. 2004). Additional evidence suggests that LANA cooperates with HIV Tat and upregulates the HIV LTR promoter, thus supporting the hypothesis that KSHV coinfection promotes HIV propagation (Hyun et al. 2001). Furthermore, LANA can also activate the major latent EBV promoter elements (LMP1 and Cp1) in transient studies (Groves et al. 2001).

KSHV prevents the cell surface expression of MHC chains and protects itself from CTL cell lysis during the lytic cell cycle. However, as only a subset of the infected cells undergo lytic replication (1%–5%), KSHV is likely to have a means to protect itself from the immune response during latent infection (Staskus et al. 1997; Zhong and Ganem 1997; Dittmer et al. 1998; Sarid et al.

1998; Katano et al. 1999; Talbot et al. 1999; Parravicini et al. 2000). Infection of primary dermal microvascular endothelial cells with KSHV resulted in downregulation of cell surface MHC class I, CD31 (PE-CAM), and CD54 (ICAM-I) immune response modulators. Moreover, this downregulation was correlated with increased levels of LANA, suggesting that during latency LANA may have a role in the downregulation of MHC class I molecules (Tomescu et al. 2003).

More recent, interesting studies have demonstrated a role for LANA in regulating KSHV reactivation, and this involves modulation of the major Notch cellular signaling pathway (Lan et al. 2005b). In our most recent studies, we demonstrated that intracellular activated domain of Notch1 (ICN) is accumulated in KSHV latently infected cells through stabilization mediated by LANA (Lan et al., unpublished data). This increase in levels of ICN is crucial for oncogenesis induced by KSHV as shown by the significant increase in proliferation of KSHV-infected cells. The ability of ICN to potentially reactivate the virus through upregulation of RTA can be effectively antagonized by KSHV LANA. This provides a finely tuned model of virus-host interaction in which KSHV selectively usurps the function of a conserved cellular signaling pathway to achieve oncogenesis while dispensing additional function to strictly maintain latency. Taken together, these studies now clearly show a feedback mechanism whereby KSHV can establish latency by repressing the expression of its major transactivator. Further work is needed to explore this new functional role of LANA. However, so far we can say with confidence that LANA plays a critical role in KSHV-mediated tumorigenesis by regulating transcription, chromatin remodeling, episomal maintenance, replication of genome, and control of reactivation and thus maintenance of KSHV latency.

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The KSHV and Other Human Herpesviral G Protein-Coupled Receptors

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1	Introduction	138
2	Overview of the KSHV vGPCR	139
3	KSHV vGPCR Signaling and Function	141
3.1	Multiple Ligands and Many Signaling Cascades	141
3.2	KSHV vGPCR and Disease Pathogenesis	143
4	Other Pathogenic GPCRs	145
4.1	Human Herpesviral GPCRs	146
4.2	Nonviral Constitutively Active GPCRs and Disease	148
5	Conclusions	149
	References	149

Abstract Kaposi sarcoma-associated herpesvirus (KSHV) is a γ 2-herpesvirus discovered in 1994 and is the agent responsible for Kaposi sarcoma (KS), an endothelial cell malignancy responsible for significant morbidity and mortality worldwide. Over time, KSHV has pirated many human genes whose products regulate angiogenesis, inflammation, and the cell cycle. One of these encodes for a mutated G protein-coupled receptor (GPCR) that is a homologue of the human IL-8 receptor. GPCRs are the largest family of signaling molecules and respond to a wide array of ligands. Unlike its normal counterpart, the mutations present in KSHV vGPCR result in constitutive, ligand-independent signaling activity. Signaling by the KSHV vGPCR results in the elaboration of many mitogenic and angiogenic cytokines that are vital to the biology of KS and other KSHV-driven malignancies. Several other herpesviruses also encode GPCRs, the functions of which are under ongoing investigation. In addition, several human diseases are associated with mutated mammalian GPCRs in germline or somatic cells.

1 Introduction

Kaposi sarcoma (KS) is a multicellular tumor consisting of spindle cells of lymphatic endothelial origin and infiltrating hematopoietic cells (Kaaya et al. 1995; Wang et al. 2004). It is a cytokine-driven process that begins as a polyclonal proliferation with a low number of spindle cells and a robust inflammatory component. Although they induce an inflammatory and angiogenic reaction, KS spindle cells are not fully transformed in that they are dependent on exogenous growth factors in culture and do not form tumors in animal models (Salahuddin et al. 1988). Over time, KS lesions may evolve into a true clonal malignancy as evidenced by X chromosome inactivation studies and comparisons of multiple lesions from a single patient (Rabkin et al. 1997). Kaposi sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8 (HHV-8), is a γ 2-human herpesvirus that was discovered in 1994 and is the etiologic agent in all epidemiologic forms of KS (Chang et al. 1994, 1996b; Boshoff et al. 1995; Dupin et al. 1995; Whitby et al. 1995). It is also present in all forms of primary effusion lymphoma (PEL) and is associated with a more aggressive phenotype of multicentric Castleman disease (MCD) (Soulier et al. 1995; Cesarman et al. 1996a; Dupin et al. 2000). Like Epstein-Barr virus (EBV), KSHV is lymphotropic; its ability to infect B cells and monocytes is vital to new virion production and to the pathobiology of all KSHV-mediated disease.

One of the first potential KSHV oncogenes described was the viral G protein-coupled receptor (vGPCR) encoded by ORF 74 (Arvanitakis et al. 1997; Guo et al. 1997). GPCRs are also known as seven-transmembrane domain receptors or serpentine receptors and are the largest family of signal-transducing molecules, estimated to have a thousand members. By some accounts, GPCRs are the target of up to 30% of all clinically available medications (Gershengorn and Osman 2001). The amino terminus of GPCRs is situated extracellularly, whereas the carboxy terminus sits intracellularly. The seven α -helices span the cell membrane and make up the transmembrane bundle. Potential ligands include neurotransmitters, growth factors, hormones, calcium ions, and photons. Binding of ligand induces a relative shift in the positions of the transmembrane α -helices. Small ligands may bind within the transmembrane bundle, whereas larger ligands bind the amino termini and extracellular loops. Ligand-induced movement of the α -helices causes conformational changes of the intracellular loops that activate the heterotrimeric GTP-binding (G) protein. Once activated, the G protein exchanges GDP for GTP and splits into two separate signaling effectors: the α -subunit and the $\beta\gamma$ -subunit. Normal G proteins contain an intrinsic

GTPase activity that converts GTP back to GDP, resulting in reassembly of the α and $\beta\gamma$ subunits. Depending on which type(s) of heterotrimeric G proteins it binds, a given GPCR can signal via phospholipase C (PLC), ion channels or adenylyl cyclase (for review, see Estep et al. 2003).

The KSHV vGPCR is different from most GPCRs in that it signals constitutively. Although its activity can be modified up or down by various ligands, it does not require an agonist to signal. Furthermore, vGPCR is a promiscuous receptor in that it binds chemokines of various families and activates a broad range of signaling cascades depending on the cellular context. The result is a complicated network of transcription factor activation affecting both cellular and viral transcription patterns. Some models suggest that vGPCR is directly transforming and can be considered a viral oncogene. Other studies argue that vGPCR influences KSHV-mediated disease in a paracrine fashion via its ability to elicit the production of various cytokines, thereby altering the tumor microenvironment in KS, PEL, and MCD. As discussed below, there is good evidence that vGPCR may play multiple roles depending on the requirements of the KSHV viral life cycle and whether it is signaling in the context of endothelial or hematopoietic cells.

2 Overview of the KSHV vGPCR

During its coevolution with its human host, KSHV has acquired many homologues of human genes involved in cell cycle regulation, angiogenesis, and inflammation (Chang et al. 1996a; Russo et al. 1996; Cheng et al. 1997; Neipel et al. 1997; Swanton et al. 1997; Wong 1998). KSHV vGPCR has the potential to affect all these processes. Like ORF 74 of murine gammaherpesvirus 68, KSHV vGPCR is a constitutively active variant of the human IL-8 receptors CXCR1 and CXCR2, as well as herpesvirus saimiri ECRF3 (Ahuja and Murphy 1993; Cesarman et al. 1996b; Arvanitakis et al. 1997; Guo et al. 1997; Wakeling et al. 2001). vGPCR was first shown to have oncogenic potential in fibroblasts, but later work also proved that vGPCR could immortalize human umbilical vein endothelial cells (HUVEC) and protect them from serum deprivation (Bais et al. 1998; Couty et al. 2001; Montaner et al. 2001). In vivo, KSHV infects cells of endothelial and hematopoietic origin. Signaling molecules can have very different effects depending on the cellular context, so these initial studies in endothelial cells were important in establishing vGPCR as a vital component of KSHV-mediated cellular proliferation.

KSHV vGPCR can influence the expression of various cytokines involved in the biology of KS and PEL. For example, KS depends on vascular en-

endothelial growth factor (VEGF) for its highly vascular morphology, and KS spindle cells secrete and respond in an autocrine manner to VEGF (Masood et al. 1997; Samaniego et al. 1998). Sodhi et al. show that vGPCR mediates VEGF secretion by stimulating the transcription factor hypoxia-inducible factor (HIF)-1 α in fibroblasts (Sodhi et al. 2000). Conditioned medium from vGPCR-expressing fibroblasts in turn stimulates endothelial cell growth and the switch to an angiogenic phenotype (Bais et al. 1998). A fascinating study by Bais et al. in HUVEC shows that vGPCR induces immortalization with constitutive VEGF receptor-2/ KDR expression and activation. This was associated with antisenescence mediated by alternative lengthening of telomeres and an antiapoptotic response (Bais et al. 2003). vGPCR expression also increases VEGF production from hematopoietic cells (Cannon et al. 2003); this is significant in that VEGF is essential for PEL tumor growth and ascites production in mice and furthermore contributes to a more aggressive phenotype of MCD (Aoki and Tosato 1999, 2001; Nishi and Maruyama 2000).

In addition to VEGF, vGPCR induces the expression of many proinflammatory cytokines important in KSHV-mediated disease. The monocytic line THP-1 expresses IL-1 β , TNF α , and IL-6, and Jurkat cells elaborate IL-2 and IL-4 when transfected with vGPCR (Schwarz and Murphy 2001). In PEL cell lines, vGPCR upregulates KSHV vIL-6, an important growth factor in PEL and MCD (Aoki et al. 1999; Jones et al. 1999; Liu et al. 2001). In endothelial cells, vGPCR upregulates the NF κ B-dependent inflammatory cytokines RANTES, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF). It also induces expression of the adhesion molecules VCAM-1, ICAM-1, and E-selectin. In the same study, supernatants from transfected KS cells activated NF- κ B signaling in untransfected cells and caused the chemotaxis of monocyte and T-lymphoid cells (Pati et al. 2001). This suggests that vGPCR may help to recruit hematopoietic cells that make up the inflammatory component of KS lesions.

Although it is directly transforming in fibroblasts, and perhaps responsible for an immortalizing autocrine loop in endothelial cells (Arvanitakis et al. 1997; Bais et al. 1998; Bais et al. 2003), the ability of vGPCR to cause such robust and broad cytokine activity argues for an important paracrine role in KSHV-mediated disease. This is supported by the first vGPCR mouse model in which vGPCR was transcribed under the CD2 promoter (primarily T cell) and resulted in multicentric, angioproliferative lesions histologically similar to KS (Yang et al. 2000). The lesions ranged from erythematous maculae to vascular tumors; they contained spindle and inflammatory cells and expressed CD34 and VEGF. Despite causing endothelial cell tumors, vGPCR was expressed from relatively few infiltrating T cells. Other mouse models and evidence for a paracrine role for vGPCR are discussed in Sect. 3.2.

KSHV vGPCR signaling may help to explain why AIDS-associated KS is a more aggressive tumor than classic or iatrogenic KS. It has been known for several years that the HIV-1 transactivator protein Tat activates KS cell growth and contributes directly to KS pathogenesis (Barillari et al. 1993; Ensoli et al. 1993; Cantaluppi et al. 2001). Pati et al. have recently shown that HIV-1 Tat and vGPCR synergistically activate the transcription factor nuclear factor of activated T cells (NFAT). In turn, NFAT activation is responsible for production of IL-2, IL-4, GM-CSF, and TNF- α s as well as expression of ICAM-1, CD25, CD29, and Fas ligand by the T cell line HUT 78 (Pati et al. 2003). The expression of these surface molecules and inflammatory cytokines results in increased endothelial cell-T cell adherence and has important implications for vGPCR as a key molecule in the interaction between KSHV and HIV-1. Indeed, later work by the same group confirmed that HIV-1 Tat increases activation of NF κ B and NFAT and accelerates tumor formation in mice that are implanted with a cell line derived from a vGPCR transgenic mouse (Guo et al. 2004).

3 KSHV vGPCR Signaling and Function

The signaling cascades that vGPCR utilizes to effect changes in viral and host cell transcription are varied and in some cases cell type specific. Despite increased understanding of vGPCR's signaling potential, correlation of these findings with biological significance remains more problematic. In this section I shall discuss what is known about the structure of vGPCR vis-à-vis its constitutive activity, the diversity of vGPCR signaling, as well as the potential roles in KSHV-mediated disease attributable to this unique receptor.

3.1 Multiple Ligands and Many Signaling Cascades

KSHV vGPCR is in the rhodopsin/ β_2 -adrenergic subfamily of GPCRs. The other two smaller subfamilies include the secretin-like receptors and the calcium-sensing/metabotropic glutamate receptors. Subfamilies are based on structural and genetic characteristics but share little intrafamilial sequence homology. Unlike normal mammalian GPCRs, vGPCR signals in the absence of ligand. This constitutive activity is largely due to abnormal transmembrane helices two and three, as well as its cytoplasmic tail (Ho et al. 2001; Schwarz and Murphy 2001). Additional mutational studies show that although the amino terminus is required for ligand binding, it is not necessary for constitutive signaling (Ho et al. 1999; Rosenkilde and Schwartz 2000). Interestingly,

despite its constitutive activity, vGPCR retains its ability to respond to various CXC and CC-type chemokines; these include agonists, inverse agonists, and neutral ligands. Agonists that increase signaling above constitutive levels include IL-8 and growth-related oncogene- α (GRO α) (Arvanitakis et al. 1997; Guo et al. 1997; Geras-Raaka et al. 1998b, 1998c; Gershengorn et al. 1998; Rosenkilde and Schwartz 2000). Neutral ligands have no effect on constitutive signaling but compete for binding, thereby inhibiting the effects of other ligands. These include neutrophil-activating peptide-2 (NAP-2) and epithelial cell-derived neutrophil-activating 78 (ENA-78) (Rosenkilde and Schwartz 2000). Inverse agonists inhibit constitutive signaling and include interferon- γ -inducible protein-10 (IP-10), stromal cell-derived factor-1 (SDF-1), and KSHV vMIP-II, a viral CC chemokine (Geras-Raaka et al. 1998b, 1998c). Of note, it is the angiogenic ELR-positive (Glu-Leu-Arg prior to the first cysteine) CXC chemokines that are vGPCR agonists, while the non-ELR chemokines are inverse agonists (Strieter et al. 1995). This has implications for vGPCR function and is discussed below. Another mechanism by which vGPCR may be modulated *in vivo* is via the GPCR-specific kinases (GRK), particularly the family containing GRK-4, -5, and -6 (Premont et al. 1995; Geras-Raaka et al. 1998a). GPCRs in an active state can be bound by GRKs that lead to interaction with arrestin and inhibition of G protein interaction. The biologic importance of GRK-mediated downregulation of vGPCR remains to be studied.

KSHV vGPCR not only binds a huge array of ligands but utilizes a surprising number of downstream effectors. These are cell type specific, so I shall concentrate on the data in endothelial and hematopoietic cells because they are the natural targets for KSHV infection. vGPCR can signal via G α_i and G α_q in both cell types (Couty et al. 2001; Cannon and Cesarman 2004). Studies in HeLa cells suggest that coupling to G α_{13} and RhoA may also be possible, but this remains to be confirmed in a biologically relevant cell type (Shepard et al. 2001). The $\beta\gamma$ -subunits of both pertussis-sensitive (G α_i) and pertussis-insensitive α -subunits also mediate vGPCR signaling (Couty et al. 2001; Montaner et al. 2001; Smit et al. 2002; Cannon and Cesarman 2004). The result is the activation of several kinases involved in endothelial and B cell proliferation. For example, vGPCR-mediated activation of a G α_i -PI3K/Akt axis promotes endothelial cell survival; this is likely via activation of NF κ B (Couty et al. 2001; Montaner et al. 2001; Pati et al. 2001). Although NF κ B is also activated in B cells, we have found that it is neither G α_i - nor PI3K/Akt dependent but rather mediated by a non-pertussis-sensitive G protein such as G α_q (Cannon and Cesarman 2004). vGPCR also activates the mitogen- and stress-activated protein kinases (MAPK, SAPK) involved in cellular proliferation, angiogenesis, and inflammation via both G α_q and G α_i G proteins (Bais et al. 1998; Smit et al. 2002; Cannon et al. 2003). This results in additional

transcription factor activation including AP-1, CREB, and HIF1- α ; the latter has been shown to result in VEGF expression from COS-7 cells and may well apply to endothelial and hematopoietic cells (Sodhi et al. 2000).

NFAT is a transcription factor that acts in conjunction with AP-1 to enhance the expression of many proteins in the productive immune response; furthermore, it is required for the calcium-mediated latent-to-lytic switch in KSHV transcription pattern (Macian et al. 2001; Zoetewij et al. 2001). We and others have shown that vGPCR induces NFAT activity via combined interaction with G α_q and G α_i ; and that this is augmented by HIV-1 Tat (Cannon et al. 2003; Pati et al. 2003). Chiou et al. discovered that vGPCR upregulates transcription via the T1.1/PAN, K1, and LLP latency promoters of KSHV, while we show a vGPCR-induced increase in the lytic products ORF 50 and ORF 57 in latently infected PEL cells (Chiou et al. 2002; Cannon et al. 2003). So, in addition to its effects on host cell transcription, it is clear that vGPCR has important effects on KSHV transcription patterns; as discussed below, this has important implications for the role of vGPCR in the KSHV viral life cycle.

3.2

KSHV vGPCR and Disease Pathogenesis

KSHV vGPCR is a promiscuous receptor that activates cell survival signaling cascades, results in the elaboration of various cytokines, and can affect KSHV transcription patterns. Any or all of these functions may be vital to the pathogenesis of KSHV-mediated endothelial and B cell hyperproliferative syndromes. Some of the first studies showed that vGPCR transforms fibroblasts (Arvanitakis et al. 1997). However, arguing for a directly transforming role in KSHV-mediated disease is difficult: Unlike the latent transforming viral genes of EBV, vGPCR is expressed in the immediate-early lytic phase (Kirshner et al. 1999; Nador et al. 2001). A lytically activated host cell is destined to die in the process of new virion production, so to express a viral oncogene under such circumstances appears at odds with promoting an autocrine survival advantage. Some have argued that early vGPCR expression in endothelial cells may set up a self-perpetuating growth-promoting paracrine loop via upregulation of VEGF and VEGF receptors (Bais et al. 2003); this is a very enticing scenario but requires further investigation.

The bulk of evidence indicates that vGPCR has its most potent effects via a paracrine mechanism. KS and PEL tumors show that a relatively small subset of cells are KSHV infected and even fewer express lytic genes such as vGPCR (Staskus et al. 1997; Teruya-Feldstein et al. 1998; Dupin et al. 1999; Linderroth et al. 1999). Likewise, vGPCR-driven KS-like tumors in mice result from vGPCR expression by a subset of tumor cells (Yang et al. 2000; Guo

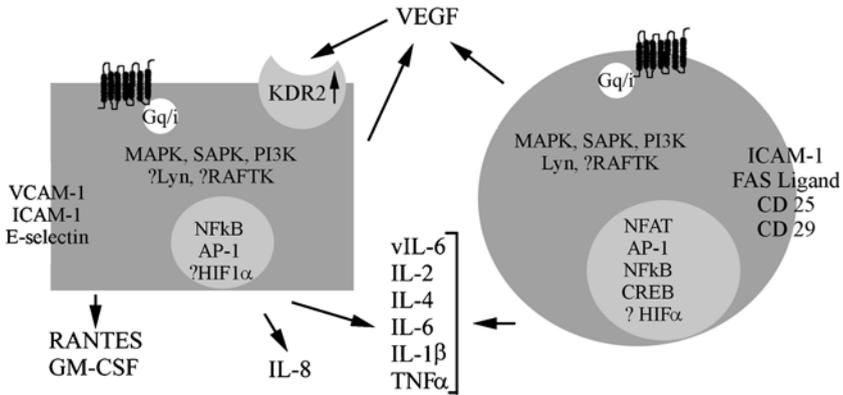


Fig. 1 Signaling intermediates, transcription factors, cytokines, and cell surface molecules upregulated by KSHV vGPCR in endothelial cells (*left*) and hematopoietic cells (*right*). In both cell types, vGPCR signals via $G\alpha_q$ and $G\alpha_i$ G proteins and activates a broad array of effector kinases that result in enhanced transcription factor activity. In addition to autocrine cell survival signals, various differentiation, angiogenic, and immunomodulatory factors are elaborated. Elements with *question marks* indicate that studies were not done in these specific cell types

et al. 2003). All KSHV-driven tumors display some level of lytic replication. Given the instability of KSHV infection in most cell types, this is probably required to maintain ongoing infection and new virion production (Lagunoff et al. 2002). When expressed from these lytically activated host cells, vGPCR could provide growth-promoting cytokine expression; and as a chemokine receptor involved in cell migration, it could also recruit new infectable cells to the tumor microenvironment. Figure 1 summarizes some of the cytokines and cell adhesion molecules upregulated by vGPCR that could explain its paracrine-driven proliferative effect on both hematopoietic and endothelial cells (also see Sect. 2).

Despite its obvious potential to affect the tumor microenvironment, if vGPCR is expressed only briefly during the early lytic phase, the question remains as to how relevant its effects are *in vivo*. However, some evidence suggests that vGPCR can be expressed in a dysregulated way, outside the normal KSHV lytic program (Sodhi et al. 2004). For example, HIV Tat can increase the expression of vGPCR, and vGPCR transcription is upregulated by RBP-J, a transcription factor and target of the Notch pathway (Yen-Moore et al. 2000; Liang and Ganem 2004). If sustained upregulation of vGPCR were to occur in an abnormal or abortive lytic phase, it would become easier to reconcile its *in vitro* potential with tumorigenesis and effects on the viral

life cycle. Interestingly, abortive lytic cycle progression in which a subset of lytic genes are expressed has been found in other herpesviruses. Dysregulated vGPCR expression would have implications for our recent work. We have shown that vGPCR signaling in PEL cells leads to a p21-mediated inhibition of cyclin-dependent kinase-2 (Cdk2) and consequent cell cycle arrest (Cannon et al. 2006). Furthermore, inhibition of Cdk2 drastically suppresses the efficiency of chemically induced reactivation of KSHV as evidenced by transcription of the lytic products ORF 50 and ORF 26. This opens up the possibility that dysregulated vGPCR expression leads to inhibition of full productive lytic replication. In this state of limbo between normal latent and lytic phases, vGPCR-mediated elaboration of angiogenic and mitogenic chemokines would have a more prolonged and biologically important effect.

Reconciling all the data on KSHV vGPCR into one well-circumscribed function is not yet possible. In fact, current concepts in virology and tumorigenesis may require us to interpret vGPCR as having multiple seemingly discrete functions. It is clear, however, that KSHV has evolved toward tight regulation of vGPCR: Its expression is restricted in that it is transcribed within the 3'-end of a bicistronic message; furthermore, KSHV encodes its own inverse agonist of vGPCR, vMIP II. Such fine-tuning of vGPCR signaling suggests that vGPCR may play different roles at different points in the KSHV life cycle. Work by Dezube et al. shows that during early de novo KSHV infection of endothelial cells, vGPCR transcription fluctuates in a cyclic pattern every 48–72 h, consistent with viral replication (Dezube et al. 2002). So, in addition to maintaining a suitable tumor microenvironment as discussed above, it may be that vGPCR has yet another role in early infection, perhaps to establish successful latency or encourage the initial rounds of KSHV replication.

4

Other Pathogenic GPCRs

Virus-encoded GPCRs have been described in several DNA viruses including poxviruses and the β - and γ -herpesvirus (Table 1). As exemplified by KSHV vGPCR, these viral GPCRs are more promiscuous in their use of ligands and downstream signaling cascades than their mammalian homologues. Such broad signaling has resulted in many postulated roles for viral GPCRs: immune evasion, viral entry and replication, cell migration, and even direct cell transformation. In addition to virally encoded constitutive GPCRs, several mutated mammalian GPCRs are also responsible for human disease.

Table 1 Human herpesvirus G protein-coupled receptors

Virus	Family	Gene	Constitutive	References
CMV	β	US27	–	Fraile-Ramos et al. 2002
		US28	+	Kledal et al. 1998
		UL33	+	Marguiles et al. 1996
		UL78	–	Bankier, DNA Seq., 1991 2;1
HHV-6	β	U12	–	Gompels et al. 1995 Isegawa et al. 1998
		U51	–	Milne et al. 2000
HHV-7	β	U12	–	Nicholas et al. 1996 Tadagaki et al. 2005
	β	U51	–	Nicholas et al. 1996 Tadagaki et al. 2005
	γ 1	BILF1	+	Beisser et al. 2005 Paulsen et al. 2005
KSHV	γ 2	ORF 74	+	Bais et al. 1998 Rosenkilde et al. 1999

4.1

Human Herpesviral GPCRs

EBV is a ubiquitous lymphotropic γ 1-herpesvirus that infects and remains latent in over 90% of people by the time they reach middle age. Manifestations of acute infection often go unnoticed in children but can cause infectious mononucleosis in adolescents and adults. EBV infects the oropharyngeal epithelium and surface B cells of the tonsils; via the latter it disseminates throughout the reticuloendothelial system. Although infection persists throughout life, the immune-competent host generally suffers no long-term ill effects after the EBV-driven polyclonal B cell proliferation subsides, thanks in part to a vigorous cytotoxic T cell response. EBV can, however, contribute to more sinister sequelae: nasopharyngeal carcinoma is most prevalent in southeastern China; Burkitt lymphoma is one of the most common childhood malignancies in sub-Saharan Africa; posttransplant lymphoproliferative disorder is EBV driven; and two-thirds of AIDS-related non-Hodgkin lymphomas are EBV positive.

Very recently the EBV open reading frame BILF1 was demonstrated to encode a functional, constitutively active GPCR that is heavily glycosylated and localizes to the plasma membrane in epithelial cell lines. The BILF1 signals via $G\alpha_i$, but not $G\alpha_{q/11}$, as evidenced by its ability to inhibit forskolin-stimulated CREB activity in a pertussis-sensitive manner in COS-7 cells. Like

KSHV vGPCR, BILF1 appears to be an immediate-early product and can activate NF κ B. Interestingly, BILF1 reduces levels of phosphorylated RNA-dependent antiviral protein kinase (PKR). PKR is an interferon-inducible enzyme with a role in intracellular antiviral defense; BILF1 is the first GPCR shown to potentially inhibit this pathway (Williams 1999; Beisser et al. 2005; Paulsen et al. 2005). Further studies of BILF1's role in the EBV life cycle and its influence over host antiviral responses are exciting prospects.

The β -herpesvirus family includes cytomegalovirus (CMV) and human herpesvirus-6 and -7 (HHV-6, -7). In healthy hosts, acute CMV infection is a self-limited flulike syndrome, and by adulthood, 70%–90% of people are latently infected. Infection during pregnancy, however, can lead to serious birth defects including blindness, deafness, seizures, and microencephaly. In the setting of AIDS, CMV causes retinitis, gastrointestinal disease, and various neurologic syndromes. In recipients of solid organ transplants, CMV causes hematologic disorders, hepatitis, pneumonitis, and organ rejection. In bone marrow transplants the more common complications are graft-versus host disease, delayed engraftment, and pneumonitis.

The CMV genome encodes four GPCRs, two of which will be discussed here: US28 and UL33. Like KSHV vGPCR, CMV US28 signals independently of an agonist but can be modified by CC chemokines and fractalkine (CX3CL1). Unlike KSHV vGPCR, US28 is located predominantly endosomally, not on the cell surface (Waldhoer et al. 2003). Expression of US28 results in migration of smooth muscle endothelial cells, and it is therefore postulated to play a role in viral dissemination and CMV-driven vascular disease (Kledal et al. 1998; Streblow et al. 1999; Casarosa et al. 2001). A role in immune evasion is also argued by virtue of the high turnover of US28 and the resultant sequestration of host-produced chemokines (Bodaghi et al. 1998). As discussed for KSHV vGPCR, another important role for US28 may lie in its latent expression in hematopoietic cells, resulting in homing to sites of inflammation rather than lymph nodes (Streblow et al. 2003). CMV UL33 also demonstrates constitutive basal activity but binds no known chemokines. It is incorporated into viral particles and expressed on virus-infected cells (Margulies et al. 1996). UL33 activates CREB-mediated signaling and because many CMV gene promoters contain CREs, it has been postulated that UL33 is involved in establishing viral infection or possibly reactivation (Casarosa et al. 2003). The rat and mouse CMV homologues of UL33 are important for replication in salivary glands and for general virulence, but similar studies are not available for CMV UL33 (Beisser et al. 1998).

Infection with HHV-6 and -7 generally occurs early in life. The former is the usual agent of exanthem subitum (also known as roseola infantum or sixth disease), a self-limited syndrome of fever and rash in children. Primary

infection later in life causes an infectious mononucleosis syndrome much like EBV or CMV. HHV-7 is not convincingly associated with disease in the normal host but, like HHV-6, may become reactivated in the immunocompromised patient (Clark et al. 2003). Two ligand-dependent GPCRs with homology to the CC-chemokine receptors are encoded by both HHV-6 and HHV-7. U12 and U51 are positional and structural homologues of CMV UL33 and UL78 (Gompels et al. 1995; Nicholas 1996; Isegawa et al. 1998; Milne et al. 2000). HHV-6 UL12 binds RANTES, macrophage inflammatory protein-1 α and -1 β (MIP-1 α , MIP-1 β), and monocyte chemoattractant protein 1 (MCP-1); HHV-7 UL12 binds EBI1-ligand chemokine (ELC), secondary lymphoid-tissue chemokine (SLC), thymus and activation-regulated chemokine (TARC), and macrophage-derived chemokine (MDC) (Nakano et al. 2003; Tadagaki et al. 2005). Both U12 homologues are functional and signal via non-G α_i pathways resulting in transient calcium flux. HHV-7 U12 induces migration of stably transfected Jurkat cells toward ELC and SLC, but not TARC or MDC. This leads the authors to postulate that HHV-7 U12 induces the migration of infected cells toward lymph nodes, perhaps facilitating viral transmission (Tadagaki et al. 2005). Little is known of the function of HHV-6 U12.

In both HHV-6 and HHV-7, UL51 is expressed in the immediate-early and early postinfection stages. Both homologs bind many CC-type chemokines as well as the KSHV-encoded homolog of MIP-II (vMIP-II). HHV-6 UL51 downregulates transcription of RANTES, which may lend it an immunomodulatory role, but this requires further confirmation. Even less is known about the function of HHV-7 UL51 (Milne et al. 2000).

4.2

Nonviral Constitutively Active GPCRs and Disease

Aside from viral GPCRs, there exist several constitutively active GPCR mutants associated with human disease that may arise in germline or somatic cells (Coughlin 1994; Spiegel 1996; Arvanitakis et al. 1998). Familial forms of hyperthyroidism and hypoparathyroidism are due to mutations in the thyroid-stimulating hormone receptor in the former and the extracellular domain of the calcium-sensing receptor in the latter (Van Sande et al. 1995; Chattopadhyay et al. 1996). The retinal degeneration of retinitis pigmentosa is thought due to mutations in the seventh transmembrane domain of rhodopsin, leading to overactivation of photoreceptor cells (Robinson et al. 1992). Mutation of the rhodopsin second transmembrane domain leads to congenital night blindness (Rao et al. 1994). A change in the sixth transmembrane helix of the luteinizing hormone receptor causes familial male precocious puberty, as do other activating mutations (Shenker et al. 1993; Kosugi et al. 1995).

5

Conclusions

Both mammalian and viral constitutively active GPCRs are associated with human disease. The study of viral GPCRs continues to teach us about viral reproduction, immune system evasion, and tumorigenesis, and even about normal GPCR function. The KSHV vGPCR is one of the best-studied and most versatile viral GPCRs. Although KSHV vGPCR alone can cause KS-like tumors in animal models, its overall role in the KSHV life cycle and biology of KSHV-driven endothelial and hematopoietic proliferative disorders is only partly understood. Undoubtedly though, its ability to affect cell growth and differentiation, cytokine production, chemotaxis, and viral transcription make it a promising target for rationally designed anti-KSHV therapies.

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Regulation of KSHV Lytic Gene Expression

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1	Introduction	158
2	Transcriptional Regulation of Immediate-Early Genes	160
2.1	Overview	160
2.2	Regulation of RTA Gene Transcription by KSHV Proteins	162
2.3	Cellular Signaling Pathways That Control RTA Expression	164
2.4	Effect of Chromatin Architecture on RTA Gene Expression	165
3	Transcriptional Regulation of Early Genes	166
3.1	Overview	166
3.2	RTA Activation of PAN and Kaposin Genes	166
3.3	RTA Activation of ORF57 and vMIP1 Genes	168
3.4	RTA Activation of IL-6 Genes	168
3.5	RTA Activation of Other KSHV Early Lytic Genes	169
4	Viral and Cellular Factors That Interact with RTA Protein	171
4.1	Overview	171
4.2	RBP-J as a Coactivator of RTA	171
4.3	Other Cellular Proteins Interacting with RTA	171
4.4	Viral Proteins Interacting with RTA	172
4.5	Other Regulators of RTA Activity	173
5	Perspectives	173
	References	175

Abstract The life cycle of KSHV, latency versus lytic replication, is mainly determined at the transcriptional regulation level. A viral immediate-early gene product, replication and transcription activator (RTA), has been identified as the molecular switch for initiation of the lytic gene expression program from latency. Here we review progress

on two key questions: how RTA gene expression is controlled by viral proteins and cellular signals and how RTA regulates the expression of downstream viral genes. We summarize the interactions of RTA with cellular and other viral proteins. We also discuss critical issues that must be addressed in the near future.

1 Introduction

Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), was discovered by its association with Kaposi sarcoma (KS), the most common form of cancer in human immunodeficiency virus type 1 (HIV-1)-infected patients (Chang et al. 1994). Subsequently, KSHV was found to be associated with two lymphoproliferative diseases related to HIV-1 infection: primary effusion lymphoma (PEL, a non-Hodgkin lymphoma) and multicentric Castleman disease (MCD) (Cesarman et al. 1995a; Soulier et al. 1995; Knowles and Cesarman 1997). The genomic DNA of KSHV (Russo et al. 1996) consists of a 140.5-kb-long unique coding region flanked by multiple 801-bp GC-rich terminal repeat sequences. The long unique coding region encodes over 80 open reading frames (ORFs), including many cellular gene homologues implicated in viral pathogenesis (Russo et al. 1996). Based on its genomic organization and other properties, KSHV has been classified as a member of the gamma 2-herpesvirus subfamily (Neipel and Fleckenstein 1999; Schulz and Moore 1999).

Like other herpesviruses, KSHV has two distinct phases in its life cycle, latency and lytic replication. Latency is characterized by persistence of the viral genome with expression of a limited set of viral genes. Once the virus is reactivated from latency and enters the lytic cycle, most viral genes are expressed in an orderly fashion (immediate-early, early, and late), leading to the production of infectious virions. KSHV gene expression patterns in latency and lytic phase have been studied with biopsies from KS/MCD/PEL, cell lines derived from PEL, and *de novo* infection of cultured cells *in vitro*. *In situ* hybridization studies of KS biopsies showed that the majority of tumor cells in the lesions contain KSHV genomic DNA and express viral latent transcripts, but in a subpopulation of tumor cells (1%–3%), viruses spontaneously enter the lytic cycle as evidenced by the expression of lytic transcripts (Staskus et al. 1997; Sun et al. 1999). Notably, these transcripts include those encoding for viral macrophage inflammatory protein-1 (vMIP-1), viral interleukin-6 (vIL-6), viral Bcl-2 homologue, as well as an unusual polyadenylated nuclear RNA (PAN). Colocalization experiments have also shown that it is the same subpopulation of cells in KS lesions that spontaneously express both the

early (e.g., PAN RNA and vMIP-1) and late [e.g., major capsid protein (MCP) and small viral capsid protein (sVCA)] viral transcripts. These observations suggest a novel paradigm for pathogenesis and tumorigenesis by an oncogenic herpesvirus. Whereas transforming function of certain viral latent gene products expressed in a majority of the tumor cells may play direct roles in tumorigenesis, production of viral and cellular cytokines by adjacent infected cells in which KSHV undergoes lytic replication may create a favorable microenvironment to enhance the growth of latently infected cells and hence also contribute significantly to tumor development.

A number of human B cell lines derived from PEL have greatly facilitated the study of KSHV gene expression, gene function, and viral pathogenesis. Similar to KS biopsies, these PEL cell lines predominantly carry the virus in a latent state, with a small subset of cells in which the virus undergoes spontaneous reactivation (Cesarman et al. 1995b; Arvanitakis et al. 1996; Renne et al. 1996; Said et al. 1996; Staskus et al. 1997; Boshoff et al. 1998; Sun et al. 1999). Viral lytic replication in the whole cell population can be further activated by treating cells with inducing chemical agents such as phorbol esters or sodium butyrate (NaB) (Renne et al. 1996; Miller et al. 1997; Nicholas et al. 1997a; Sarid et al. 1998). With these cell lines (e.g., BC-1, BCBL-1, and BC-3), KSHV gene expression patterns have been more extensively examined through Northern blot analysis (Zhong et al. 1996; Sarid et al. 1998; Sun et al. 1999). These studies showed that the kinetics of KSHV gene expression is similar to those of other herpesviruses, that is, viral gene expression is restricted in latency, and lytic genes are expressed in a cascade fashion (immediate-early, early, and late), leading to the production of infectious virions (Renne et al. 1996; Vieira et al. 1997; Gradoville et al. 2000).

More recently, the temporal pattern of KSHV gene expression has been examined on a genomic scale with custom-built DNA microarrays (Jenner et al. 2001; Paulose-Murphy et al. 2001). These studies confirmed previous results on the kinetics of viral gene expression and identified a correlation between stages of gene expression and the function of the gene in viral replication. For instance, early genes such as those encoding proteins involved in viral DNA replication are expressed at relatively early time points, whereas those involved in virion assembly are expressed at later time points. Gene expression regulation of Epstein-Barr virus (EBV) has been extensively studied, providing information that is instructive for KSHV studies. In this review, we mainly discuss some of the regulatory steps in KSHV lytic gene expression and their implications in viral replication and pathogenesis.

2 Transcriptional Regulation of Immediate-Early Genes

2.1 Overview

Immediate-early genes are the first group of genes expressed during herpesvirus lytic replication (de novo infection or reactivation) and are usually defined by their transcription without requiring de novo protein synthesis. They generally encode regulatory proteins that either activate the downstream viral lytic gene expression cascade and/or modulate host cellular environment to facilitate viral replication. In early studies, because of a lack of an efficient permissive cell system for de novo infection, KSHV lytic gene expression was generally studied by treating latently infected PEL cell lines with chemical inducers, such as TPA or NaB, to activate the viral lytic cycle. Recently, studies on viral transcription in infected TIME, HFF cells, and 293 cells have been initiated, which provide additional information (Moses et al. 1999; Krishnan et al. 2004; Sharma-Walia et al. 2005). With chemical inducers and the protein synthesis inhibitor cycloheximide, several immediate-early genes of KSHV have been identified (Sarid et al. 1998; Sun et al. 1998; Lukac et al. 1999; Zhu et al. 1999; Gradoville et al. 2000; Haque et al. 2000; Saveliev et al. 2002). These include ORF50, ORF45, ORF K4.2, and a 4.5-kb mRNA species that corresponds to the viral genomic region between nt. 49419 and 54688. ORF K5 has also been reported as an immediate-early gene, even though its transcription is only resistant to cycloheximide at a low concentration (10 µg/ml) and its promoter is moderately activated (~5-fold) by another immediate-early protein, RTA (Haque et al. 2000).

The best-characterized immediate-early gene is RTA (replication and transcription activator, a homologue of EBV RTA, also called regulator of transcription activation, ART, Lyta, or ORF50). The major transcript is a 3.6-kb multiply spliced bicistronic message containing ORF50 and K8 (Sun et al. 1998; Zhu et al. 1999). The RTA protein is mainly encoded within ORF50. A splicing event upstream of ORF50 introduces a new methionine initiation codon and adds a coding region for an additional 60 amino acids (aa) to the N terminus of ORF50. The resulting RTA protein of 691 aa has a N-terminal DNA binding and dimerization domain, a C-terminal activation domain, and two nuclear localization signals (Lukac et al. 1998; Sun et al. 1998). The splicing event as well as the domain organization are highly conserved among the RTA homologues of gamma-herpesviruses including EBV and herpesvirus saimiri (HVS). The strongest homologous sequence with other RTA is found in between residues 103 and 202 within the DNA binding and dimerization domain (Sun et al. 1998). The activation domain also shows limited sequence

conservation with other RTA homologues as well as several cellular and viral transcription factors such as the herpes simplex virus-1 VP16 (Lukac et al. 1998). RTA protein has a predicted molecular mass of 73.7 kDa; however, when it is expressed in mammalian cells its migration pattern on Western blot reveals an apparent molecular mass of approximately 110 kDa, suggesting posttranslational modifications (Lukac et al. 1999; Song et al. 2002). Indeed, the RTA protein sequence contains numerous potential sites for phosphorylation. However, whether and how these sites are utilized and what other modification mechanisms are responsible for the observed increase of RTA molecular mass remain to be investigated.

Several lines of evidences have demonstrated that RTA serves as the “molecular switch” for KSHV life cycle. Expression of RTA alone in latently infected PEL cells disrupted latency and activated the expression of viral lytic genes such as vIL-6, PAN RNA, and ORF59; induction of viral late protein synthesis, ORF65 and K8.1, by RTA indicated that RTA drives the lytic cycle to completion (Lukac et al. 1998; Sun et al. 1998). Moreover, ectopic expression of RTA increased the production of DNase-resistant encapsidated viral DNA, providing the ultimate proof that RTA is capable of initiating and driving the complete viral lytic cycle, leading to the release of newly produced viral particles (Gradoville et al. 2000). On the other hand, introduction of dominant-negative mutant RTA proteins that lacked the C-terminal activation domain into latently infected cells reduced spontaneous viral reactivation, suggesting that RTA function is necessary for lytic reactivation (Lukac et al. 1999). A similar conclusion was reached by using the ribozyme approach to inhibit RTA expression (Zhu et al. 2004). Finally, deletion of RTA generated by the KSHV BAC system further confirmed the essential roles of RTA in viral lytic reactivation (Xu et al. 2005). Taken together, these data have demonstrated that RTA is both necessary and sufficient to mediate the switch from latency to lytic replication of KSHV *in vitro*. Because of the lack of an animal model for KSHV infection, several groups utilized a murine gammaherpesvirus-68 (MHV-68) to study the functions of RTA *in vivo*. The results suggested that RTA of MHV-68 also plays a central role in initiating the lytic replication cycle during infection of mice (Wu et al. 2001; Pavlova et al. 2003; Boname et al. 2004; Rickabaugh et al. 2004).

Because RTA serves as the molecular switch for viral life cycle, activation of the RTA promoter becomes the key to understanding the mechanisms controlling KSHV latency and reactivation. The activity of the RTA promoter on the viral genome should be determined by a number of factors: activators and repressors (both viral and cellular) as well as chromatin structures of the viral genome. The remainder of this section will be devoted to discussing the recent progress in understanding the transcriptional regulation of this key molecule.

2.2

Regulation of RTA Gene Transcription by KSHV Proteins

As a powerful transcriptional activator, RTA activates the expression of many viral genes, including itself (Seaman et al. 1999; Deng et al. 2000; Gradoville et al. 2000). Autoactivation of the immediate-early RTA gene represents an important strategy for KSHV to effectively respond to environmental stimuli and maximally activate the virus lytic cycle. A 0.5-kb RTA promoter sequence was activated 2.7-fold by RTA expression (Seaman et al. 1999). With a construct with a much longer upstream sequence, it was shown that a 3-kb RTA promoter is highly responsive to TPA and NaB. In addition, RTA autoactivated the 3-kb promoter reporter construct up to 144-fold, independent of other viral factors or B cell-specific factors. Furthermore, ectopic expression of RTA in latently infected cells activated the expression of the RTA gene from endogenous viral genomes as demonstrated by either ribonuclease protection assay of the 5' untranslated region (Deng et al. 2000) or Northern blot analysis of the RTA bicistronic transcripts using a probe derived from the ORF K8 region (Gradoville et al. 2000).

Several studies were conducted to map the RTA response element (RRE) in RTA promoter. A luciferase reporter with the 950-bp fragment upstream of the RTA coding region was weakly activated four- to eightfold by RTA, and the response element was mapped to an octamer-binding site (Sakakibara et al. 2001). Electrophoretic mobility shift assay (EMSA) showed that cellular Oct1 protein binds to an octamer-binding site; however, an RTA-Oct-1 complex was not observed, suggesting that the RTA protein may not associate with the octamer-binding site strongly and therefore an indirect mechanism may be involved. Nevertheless, this 950-bp promoter supports only mild activation by RTA (up to 8-fold) in comparison to a 3-kb promoter (Deng et al. 2000) that can be highly activated (144-fold), indicating that other unidentified RREs may exist further upstream. Sequence analysis of this region reveals multiple binding sites for the cellular transcription factor RBPJ. Using a luciferase construct with 3-kb promoter sequences, Liang et al. revealed that autoactivation of this RTA promoter is significantly lowered in RBPJ-null cells (10-fold) but much stronger in wild-type cells (up to 70-fold) (Liang and Ganem 2003). These observations suggested that RBPJ, a known cellular partner of RTA (Liang et al. 2002) (see more details below), is involved in the autoactivation of RTA promoter. Indeed, Chang et al. have shown that a DNA-binding-defective RTA mutant is still competent to induce the ORF50 transcription *in vivo* and that the RTA promoter with multiple RBPJ-binding sites was autoactivated at a higher level than that lacking RBPJ-binding sites (Chang et al. 2005c). These lines of evidence suggested that autostimulation

of RTA promoter is mediated mainly through indirect non-RTA-binding mechanisms and involves at least two cellular proteins, RBPJ and Oct-1.

Several viral proteins have been reported to inhibit the expression of RTA (Cannon et al. 2006). Interestingly, RBPJ-binding sites are also involved in the repression of RTA expression by latency-associated nuclear antigen (LANA). LANA is a multifunctional protein that is essential for latency establishment and maintenance. LANA mediates viral episomal DNA persistence during latency by tethering the viral episomes to cellular mitotic chromosomes (Ballestas et al. 1999; Hu et al. 2002; Fejer et al. 2003; Grundhoff and Ganem 2003). It also promotes cell survival and regulates cell cycle progression by modulating various cellular targets, including p53, Rb, and Wnt pathways (Friborg et al. 1999; Fujimuro and Hayward 2003; An et al. 2005). More recently, it has been shown that LANA suppresses lytic reactivation by repressing not only the basal level expression of the RTA promoter but also RTA-mediated autoactivation (Lan et al. 2004). Intriguingly, LANA-mediated repression of RTA promoter depends on the RBPJ-binding sites (Lan et al. 2005) that are also involved in RTA autoactivation (see above). By directly interacting with RBPJ protein, LANA may not only recruit additional corepressors to suppress the transcription of the RTA gene but also repress RTA autoactivation activity by competing with RTA in RBPJ-binding. The fact that both positive and negative regulators of RTA gene expression use the same RBPJ-dependent mechanism suggests that the switch between latency and lytic reactivation is finely controlled by the levels of LANA and RTA proteins in virus-infected cells. Furthermore, RTA also induces LANA expression (Lan et al. 2005; Matsumura et al. 2005), providing a negative feedback in keeping viral lytic reactivation under check.

Although RTA expression is strictly controlled in latency, RTA and a subset of lytic genes are transiently expressed very early after viral entry and quickly replaced by latent gene expression (Krishnan et al. 2004). The mechanisms and physiological significance for such transient expression and repression are still unknown. One possibility is that some viral factors are directly brought into the infected cells in the form of virion proteins to effectively modulate the intracellular environment to facilitate viral replication, as suggested by data from different perspectives (Lu et al. 2005). An advantage of bringing viral regulatory factors into infected cells as virion-associated proteins is that these factors can directly interact with the host cellular environment as soon as they are delivered into the cells by virus entry, independent of transcription and translation. Alphaherpesviruses and betaherpesviruses are known to incorporate a number of regulatory gene products as tegument proteins. For example, herpes simplex virus-1 virion carries transcription factor VP-16 and virion host shut-off (VHS) proteins. However, little is known about the physiological

functions of any gammaherpesvirus virion proteins immediately after infection of the cell. RTA turns out to be one of the KSHV virion-associated proteins (Bechtel et al. 2005; Lan et al. 2005). It may be involved in triggering transient expression of some lytic genes that modulate the host cell for the benefit of viral infection and/or induce the expression of latency proteins (Lan et al. 2005; Matsumura et al. 2005) that eventually shut down the lytic gene expression and establish latency in some types of cells. Further studies are needed to address the roles of KSHV virion proteins in establishing viral infection.

2.3

Cellular Signaling Pathways That Control RTA Expression

A critical question that must be addressed in KSHV biology is what host cellular signals control RTA expression and thus the balance between viral latency and lytic replication. Chemical agents such as phorbol esters, NaB, 5'-azacytidine, and glycyrrhizic acid can reactivate KSHV in latently infected cells in culture (Cesarman et al. 1995b; Miller et al. 1996; Renne et al. 1996; Chen et al. 2001; Curreli et al. 2005). These agents, although not necessarily natural physiological inducers, provide valuable tools for studying the mechanism of KSHV reactivation and may reveal potential cellular pathways that may be involved. Phorbol esters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) can activate protein kinase C (PKC) (Castagna et al. 1982), suggesting that signaling pathways initiated by PKC may contribute to physiologically relevant KSHV reactivation. Experiments of overexpression and selective inhibition indicated an essential but not sufficient role for PKC δ isoform in KSHV reactivation (Deutsch et al. 2004). In addition, activation of Ap-1 pathway has also been suggested to be involved in TPA-induced KSHV reactivation (Wang et al. 2004). A different group of chemical inducers such as NaB and 5'-azacytidine implicates the roles of chromosomal modulation in KSHV reactivation, which is discussed in Sect. 2.4.

Another approach to define the mechanism controlling the reactivation is to explore suspected physiological stimuli, such as inflammatory cytokines (Chang et al. 2000; Mercader et al. 2000; Wang et al. 2005) and hypoxia (Haque et al. 2003). Hypoxia has been proposed to be at the location where KS occurs frequently. Several neuron transmitters associated with stress responses have also been found to reactivate KSHV. One example is that epinephrine and norepinephrine efficiently reactivated lytic replication of KSHV in latently infected PEL cells via β -adrenergic activation of the cellular cyclic AMP/protein kinase A (PKA) signaling pathway (Chang et al. 2005b).

Many cellular signaling pathways have been found to positively regulate herpesvirus reactivation, but negative regulators have been less defined. It

has been found that the cellular transcription factor NF- κ B, which is highly active in lymphocytes, is required for maintaining viral latency of KSHV and EBV (Brown et al. 2003, 2005; Chang et al. 2005b). Downregulation of NF- κ B resulted in viral reactivation in latently infected B cell lines. On the other hand, overexpression of NF- κ B in epithelial or fibroblast cells inhibited MHV-68 lytic replication and allowed the virus to establish persistent infection. NF- κ B also inhibited the activation of viral lytic genes by RTA in reporter assays. The inhibition was also reversible by I- κ B and regulated by the relative amount of RTA versus NF- κ B in the cell. These data suggest that high levels of NF- κ B activity in lymphocytes play a direct role in the establishment and maintenance of viral latency in these cells. NF- κ B can be upregulated by a viral latent protein, ν FLIP (An et al. 2003; Guasparri et al. 2004). In addition, MTX was found to inhibit the lytic cycle of KSHV (Curreli et al. 2002). Interestingly, MTX, a well-known DHFR inhibitor, acts in a DHFR-independent fashion in this case. The underlying mechanism is not clear, but potentially bears novelty. The potential link between transcriptional regulation and signaling originating from cellular metabolism is a topic to be explored.

2.4

Effect of Chromatin Architecture on RTA Gene Expression

It is well known that both DNA methylation and histone acetylation play critical roles in gene regulation through chromatin remodeling (Wu and Grunstein 2000). Not surprisingly, expression of the RTA gene on the KSHV genome is also regulated at the chromatin level. Treatment of BCBL-1 cells with 5-azacytidine, a DNA methyltransferase inhibitor, induced KSHV reactivation. Bisulfite genomic sequencing analysis confirmed that the RTA promoter region is methylated and the promoter of a latent gene LANA lacks methylation in latently infected cells. These results suggested that methylation status is critical for controlling the RTA promoter activity, and hence viral reactivation (Chen et al. 2001).

In addition to DNA methylation, the chromatin structure of the RTA promoter is also regulated by histone acetylation. Positively charged histones bind tightly to the phosphate backbone of DNA, keeping chromatin in a transcriptionally silent state. Acetylation of histones by histone acetylase (HAT) neutralizes the positive charges on histones and therefore disrupts the higher-order chromatin structures for easy access by transcription factors and RNA polymerase complex to initiate transcription. Histone deacetylation mediated by histone deacetylases (HDACs) restores a positive charge on histones, leading to a tightly supercoiled chromatin structure that is associated with transcription repression. It has been known that inhibitors of HDACs such as

NaB and trichostatin A can induce lytic reactivation of the KSHV in latently infected PEL cell lines (Riggs et al. 1977; Cousens et al. 1979; Miller et al. 1996). The responsive element was mapped to a GC box that binds Sp1/Sp3, over which transcriptional initiation site a nucleosome is located (Lu et al. 2003). In latently infected cells, the RTA promoter is associated with multiple HDACs (including HDACs 1, 5, and 7), whereas NaB treatment resulted in the rapid binding of the SWI/SNF chromatin remodeling complex. Cyclic AMP-response element binding protein (CREB)-binding protein (CBP) HAT was also shown to stimulate RTA transcription from a plasmid (Lu et al. 2003). Taken together, these studies suggest that chromatin remodeling of the RTA promoter region, including histone acetylation and DNA demethylation, is a critical step in the switch from latency to lytic reactivation.

3

Transcriptional Regulation of Early Genes

3.1

Overview

KSHV early genes generally encode proteins that are involved in nucleic acid metabolism and modulation of cellular functions. They are usually under the control of the immediate-early proteins. A number of KSHV early lytic genes have been shown to be activated by RTA through direct or indirect mechanisms. They include PAN RNA (also called nut-1 or T1.1), Kaposin (Kpsn; also called K12), ORF57 (a posttranscriptional activator), K-bZIP (the KSHV homologue of ZEBRA encoded by EBV; also called K8), vIL-6, K5, K9 [viral interferon (IFN) regulatory factor or vIRF], K14 (viral OX-2), K15, ORF6 (single-stranded DNA binding protein), ORF59 (DNA polymerase-associated processivity factor), ORF21 (viral thymidine kinase or vTK), ORF74 (viral G protein -coupled receptor) (Zhang et al. 1998; Chang et al. 2000; Chen et al. 2000; Haque et al. 2000; Duan et al. 2001; Jeong et al. 2001; Lukac et al. 2001; Song et al. 2001; Deng et al. 2002b; Wong and Damania 2006). Interactions of RTA with some of these viral promoters have been characterized in detail. In this section, we will use examples to illustrate how RTA differentially activates the downstream gene expression through various mechanisms.

3.2

RTA Activation of PAN and Kaposin Genes

PAN RNA is a novel 1.1- to 1.2-kb noncoding polyadenylated transcript, forming a speckled pattern in the nucleus and colocalizing with cellular Sm pro-

tein. Therefore, PAN RNA possesses features of both U snRNA and mRNA (Sun et al. 1996; Zhong and Ganem 1997). It is the most abundant transcript expressed during KSHV lytic phase, comprising approximately 80% of the total polyadenylated RNA in infected cells. Although its function in KSHV replication and pathogenesis has remained a mystery, PAN RNA serves as a good model to define the mechanism of RTA activation. With a transient transfection reporter assay, it was demonstrated that RTA activates the PAN promoter up to 7,000-fold and that this activation is independent of other KSHV or B cell-specific factors (Song et al. 2001). Deletion analysis further mapped the RTA-responsive element (RRE) to a 31-bp region of the PAN promoter. EMSAs showed that RTA directly binds to the PAN RRE and forms a highly stable complex (Song et al. 2001). Independently, Chang et al. also mapped PAN RRE to a 25-bp region (Chang et al. 2002), which is contained within the 31-bp RRE mapped by Song et al. previously (Song et al. 2001). A detailed analysis of specific interactions between RTA and the PAN promoter showed that RTA has a strong affinity for the RRE in the PAN promoter, which is reflected in the K_d at the nanomolar range. In addition, the minimal length for RTA binding was mapped to a 30-bp region spanning from -74 to -45 of the PAN promoter (with transcription initiation site of PAN RNA at nt 28667 on KSHV genome set as +1). Results from methylation interference assay, deletion analysis, and extensive mutagenesis study using both reporter assays and EMSAs correlated with one another and revealed base pairs critical for both RTA binding in vitro and RTA transactivation in vivo (Song et al. 2002). These studies were performed with the N-terminal half of RTA protein purified from *Escherichia coli* because the full-length protein was less efficient in binding, a problem that hindered many investigators. Only recently has the Miller lab overcome the hurdle by discovering that the C-terminal end of RTA inhibits the DNA binding activity (Chang and Miller 2004).

A sequence analysis of the KSHV genome revealed that a region in Kaposin promoter bears remarkable homology to the PAN RRE sequence. The Kaposin gene is abundantly expressed during latency and strongly induced during the viral lytic cycle. Kaposin A (K12) was reported to possess cellular transformation ability (Muralidhar et al. 1998, 2000; Kliche et al. 2001). Kaposin B increases the production of cytokines via activating the p38/MK2 pathway that is known to stabilize the AU-rich element-containing transcripts as seen for most cytokines (Sadler et al. 1999; McCormick and Ganem 2005). This 25-bp homologous region in the Kaposin promoter contains a consecutive stretch of 16-bp matches and additional 5-bp matches downstream. When tested in EMSA, this region conferred binding to RTA, although at a lower affinity. Mutation of two nucleotides (CC→TG) so that the mutated Kaposin promoter sequence (Kpsn/TG) has 19 consecutive matches plus additional

4-bp matches showed that its binding affinity for RTA is higher than that of the Kaposin promoter but still lower than that of the PAN promoter (Song et al. 2003). This result indicated that the sequences downstream of the 16-bp homology are also important for binding by RTA, consistent with the previous analysis of the PAN promoter (Song et al. 2003). Deletion analyses of the Kaposin promoter sequences have further confirmed that the 16-bp homologous region is essential for RTA binding and *trans*-activation and that the 5' and 3' flanking sequences also play important roles (Chang et al. 2002).

3.3

RTA Activation of ORF57 and vMIP1 Genes

ORF57 encodes a posttranscriptional regulator that is conserved in herpesviruses. Its expression is upregulated by RTA expression (Kirshner et al. 1999, 2000; Duan et al. 2001). Using luciferase reporter assays, Lukac et al. identified a core 25-bp region in the ORF57 promoter that is responsive for RTA activation (Lukac et al. 2001). When Liang et al. first discovered the protein-protein interactions between RTA and a cellular DNA-binding factor RBPJ, they noticed an authentic RBPJ-binding site GTGGGAA within this 25-bp ORF57 RRE and showed that mutation of this site almost completely abolished RTA activation (Liang et al. 2002). Furthermore, RTA fails to activate ORF57 promoter in RBPJ-null cells while such activation can be restored by cotransfection of an RBPJ-encoding vector (Liang et al. 2002). These observations clearly demonstrated that RTA activation of ORF57 gene mainly depends on the cellular protein RBPJ bound to the RBPJ-binding site within the identified 25-bp RRE. This conclusion was further supported by a recent study from the Miller lab (Chang et al. 2005c), which showed that RBPJ rather than RTA binds ORF57 promoter in EMSA and that DNA-binding-deficient mutants of RTA can still activate ORF57 promoter but not promoters of PAN and Kaposin. Moreover, an RRE with similarity to that of ORF57 was identified in the promoter of vMIP-I (a virus-encoded chemokine homologue), and studies using EMSA and mutational analysis demonstrated that it too contains a real RBPJ-binding site and that RTA activates vMIP-I through protein-protein interaction with the bound RBPJ protein (Chang et al. 2005c).

3.4

RTA Activation of IL-6 Genes

KSHV encodes homologues of several cellular cytokines and chemokines, one of which is viral IL-6 (vIL-6). vIL-6 is encoded by ORF K2 and shares 25% amino acid identity with human IL-6. Similar to its human counterpart, vIL-6

promotes growth and proliferation of IL-6-dependent human and murine hybridoma B cell lines (Moore et al. 1996; Nicholas et al. 1997b). vIL-6 also activates multiple signal transduction pathways, including JAK/STAT and Ras-MAP kinase pathways (Molden et al. 1997; Osborne et al. 1999). The vIL-6 transcript is one of the most abundant viral mRNAs expressed during lytic phase in PEL-derived cells (Sun et al. 1999), and its expression is under the control of RTA as well (Deng et al. 2002b). Through reporter assays and EMSAs, the vIL-6 RRE has been mapped to a 26-bp region, which, however, bears no similarities to either the PAN RRE or the ORF57 RRE.

Defining the vIL-6 transcription unit has also revealed the presence of two transcriptional initiation sites, and hence two promoters (Deng et al. 2002b). The existence of two promoters is intriguing, especially in light of the variable vIL-6 levels observed in KS, PEL, and MCD samples. In situ hybridization and immunohistochemistry studies demonstrated that vIL-6 is expressed at higher levels in PEL and MCD than in KS, and the average levels of vIL-6 expressed in individual infected PEL and MCD cells are greater by at least an order of magnitude than those in KS cells (Staskus et al. 1999). The presence of two promoters allows for differential regulation of vIL-6 gene expression in cell- and tissue-specific environments, which may lead to different manifestations in distinct malignancies associated with KSHV. In this aspect, it is interesting to note that the human cellular IL-6 (hIL-6) gene is also strongly upregulated by RTA (Deng et al. 2002a). It is remarkable that the virus takes multiple approaches to upregulate and maintain IL-6 levels during latency and lytic replication, which involve the roles from v-FLIP, RTA, and Kaposin, as well as encoding a viral homologue of IL-6. This strongly implicates the essential roles of IL-6-related functions in KSHV life cycle.

3.5

RTA Activation of Other KSHV Early Lytic Genes

A virally encoded G protein-coupled receptor (vGPCR) plays important roles in KSHV-induced angiogenesis. The most abundant vGPCR-containing transcripts are bicistronic RNAs with K14 (Nador et al. 2001) at the 5' end and vGPCR at the 3' end. This K14/vGPCR transcript is strongly induced during lytic reactivation (Kirshner et al. 1999, 2000; Nador et al. 2001; Chiou et al. 2002). The promoter governing this transcript is highly responsive to RTA activation, in which three putative RREs (sites A, B, and C) were identified through deletion and mutation mapping (Liang and Ganem 2004). None of these sites binds directly to RTA; rather, both sites A and C bind to RBP-J, whereas site B binds to yet-unknown cellular factors. The importance of RBP-J in the transcription of K14/vGPCR mRNAs is demonstrated by the observa-

tion that RTA activation of K14/vGPCR promoter is significantly inhibited in cells lacking RBP-J (Liang and Ganem 2004). Interestingly, a recent study (Matsumura et al. 2005) has shown that RTA uses these same RREs to activate the latency transcript (LT) locus, encoding LANA (ORF73), v-cyclin (ORF72), and v-FLIP (ORF71), which are transcribed in the opposite direction of K14/vGPCR. Another study (Zhang et al. 2005), however, has shown that RTA can directly bind to and activate through IFN-stimulated response element (ISRE) and that one such ISRE is present within the K14/vGPCR promoter, partially overlapping site A (Liang and Ganem 2004). RTA was further shown to selectively induce some cellular IFN-responsive genes (IRGs) including ISG-54, MxA, and STAF50 (Zhang et al. 2005), whose *in vivo* functions in viral replication and pathogenesis wait further studies.

The KSHV K-bZIP (KSHV basic leucine zipper) or K8 protein is a homologue of the EBV ZEBRA (Zta), one of the two immediate-early proteins that control the life cycle of EBV. However, the KSHV K-bZIP is an early gene, whose expression is activated by RTA (Sun et al. 1998; Lin et al. 1999) and has been shown to play an essential role in viral DNA replication (Lin et al. 2003) and cell cycle regulation. Wang et al. reported that K8 gene is differentially transcribed during immediate-early (IE) and delayed-early stages with different promoters (Wang et al. 2004). K8 IE promoter can be activated by butyrate, possibly through Sp1-binding sites. K8 delayed-early promoter is activated by RTA, the mechanism of which has been characterized in detail by several groups (Lukac et al. 2001; Seaman and Quinlivan 2003; Wang et al. 2003, 2004). A total of three RREs were identified. RRE-I, via cooperative binding of RTA, RAP (K-bZIP), and cellular factor C/EBP α , has a minor effect in B cells but more in 293 cells. RRE-II plays a major role via a non-DNA-binding mechanism in both 293 and B cells, the details of which are yet to be characterized. RRE-III contains a standard RBPJ-binding site, mutation of which causes a 2.5-fold transcriptional reduction in both cell types.

KSHV vIRF (K9) has differential transcription patterns during latency and lytic phase (Chen et al. 2000). Its lytic promoter is highly responsive to RTA activation, and the RREs were finely mapped to two regions that have no sequence homology and cannot directly bind RTA *in vitro* (Ueda et al. 2002). Multiple cellular factors were found to bind to these elements, and their binding correlated with the RTA responsiveness, suggesting an indirect mechanism for RTA to act through cellular factor(s) (Ueda et al. 2002). The response of the viral *tk* promoter to RTA appears to require Sp1-binding sites (Zhang et al. 1998) and also the RBPJ protein (Liang unpublished data).

4 Viral and Cellular Factors That Interact with RTA Protein

4.1 Overview

RTA must function through interactions via other proteins. Multiple viral and cellular proteins have been identified to interact with RTA, which is consistent with the observation that RTA is a multifunctional protein. However, the challenge is to define the biological relevance of such interactions. Current progress is summarized here.

4.2 RBP-J as a Coactivator of RTA

One of the best-characterized RTA-interacting cellular proteins is the DNA-binding transcription factor RBP-J (Liang et al. 2002). RBP-J is a downstream target of the cellular Notch signal pathway. It recognizes the specific DNA sequence GTGGGAA and acts as transcriptional repressor by recruiting a corepressor complex. On activation, the Notch molecule spanning the cell membrane is cleaved to release the intracellular domain, which translocates to the nucleus, where it binds RBP-J, replaces the corepressor complex, and activates the target promoters using its intrinsic activation domain. Viral proteins such as EBV EBNA2 and KSHV RTA have been shown to pirate this cellular pathway in order to activate target promoters containing RBPJ-binding sites. Like Notch molecule and EBNA2, RTA also binds to the central repressor domain of RBP-J, and, by competing off the corepressor complex, activates target promoters using its strong activation domain. So far the mechanism of RBPJ-dependent RTA activation has been utilized by multiple RTA target promoters, such as ORF57, TK, K14/vGPCR, RTA, K3, K5, vIL-6, and vMIP-1 (Liang et al. 2002; Liang and Ganem 2003, 2004; Chang et al. 2005a, 2005c; Lan et al. 2005). The biological functions of RBP-J in the KSHV life cycle have been evaluated with a mouse RBPJ-knockout cell line (Liang and Ganem 2003), in which KSHV can still establish latency but RTA-induced lytic reactivation is completely abolished. This is likely caused by the defective expression of multiple lytic genes that depend on RBPJ for activation by RTA.

4.3 Other Cellular Proteins Interacting with RTA

Other RTA-interacting cellular transcription factors include CCAAT/enhancer-binding protein- α (C/EBP α) (Wang et al. 2003) and STAT3 (Gwack

et al. 2002). C/EBP α is a member of the leucine zipper family of transcription factors. Potential C/EBP α -binding sites are found in the promoters of K-bZIP, PAN, ORF57 (MTA), and RTA, whose activations can be cooperatively activated by RTA and C/EBP α (Wang et al. 2003). STATs are a family of proteins that is phosphorylated and activated by signaling pathways of various cytokines and growth factors. Activated STATs form homo- or heterodimers and translocate to the nucleus, where they bind to specific DNA response elements. Gwack et al. showed that interaction between RTA and STAT3 leads to STAT3 dimerization and nuclear translocation in the absence of phosphorylation and that RTA is able to induce STAT3-mediated transcription (Gwack et al. 2002). However, the biological roles of STAT3 in KSHV replication and pathogenesis have not yet been characterized.

Using GST pull-down combined with mass spectrometry, Gwack et al. identified multiple cellular transcription cofactors that can interact with RTA, including the CBP HAT, SWI/SNF complex, and TRAP/Mediator complex (Gwack et al. 2003). These cofactors interact with the RTA activation domain and presumably function to modulate chromatin structures of viral promoters and recruit general transcription factors to initiate viral gene expression. RTA also interacts with the cellular poly(ADP-ribose) polymerase I (PARP-1) and a human homologue of Ste20-related kinase from chicken (hKFC) (Gwack et al. 2003) through its serine/threonine-rich region. It was shown that PARP-1 can poly(ADP)-ribosylate RTA and hKFC can phosphorylate RTA (Gwack et al. 2003). Both PARP-1 and hKFC repress RTA transcriptional activity *in vitro* and suppress RTA-mediated lytic reactivation *in vivo*. The KSHV RTA-binding protein (K-RBP, or MGC2663) contains potential zinc finger domains and was shown to cooperate with RTA in activation of target genes such as ORF57, K8, and vMIP-1 (Wang et al. 2001). Using nuclear extracts for EMSA, Chang et al. showed that cellular proteins such as YY1 and Sp1 also bind to the sequences within the identified RREs in the promoters of PAN, Kpsn, ORF57, and vMIP-I genes (Chang et al. 2002, 2005c; Chang and Miller 2004). However, whether these cellular proteins contribute to RTA activation of the respective promoters and, if so, whether they act by protein-protein interactions with RTA remain to be studied.

4.4

Viral Proteins Interacting with RTA

In addition to cellular factors, RTA also interacts with many viral proteins that function to modulate its activity. We have described that viral latency protein LANA interacts with RTA and antagonizes its autoactivation activity and thus keeps lytic reactivation under check (Sect. 2.2). A viral early protein KbZIP was

shown to interact directly with RTA and to repress RTA activation in promoter-dependent manner, that is, it suppresses RTA activation of promoters of ORF57 and KbZIP but not of PAN. There is evidence suggesting (Izumiya et al. 2005) that sumoylation plays an important role in KbZIP's transcriptional repression activity. KbZIP represses RTA activation of a subset of promoters, which was suggested to contribute to the highly controlled expression of viral lytic genes. However, the significance of such control during viral infection is not yet understood. Another viral early protein, ORF57 (MTA), recently shown to interact with RTA *in vitro* and *in vivo* (Malik et al. 2004), cooperates with RTA to enhance the expression of target genes at a posttranscriptional level (Kirshner et al. 2000). The recent finding that RTA contains ubiquitin E3 ligase activity and targets interferon regulatory factor 7 (IRF-7) suggested a novel role of RTA in downregulating immune responses (Yu et al. 2005).

4.5

Other Regulators of RTA Activity

In addition to binding RTA directly, some regulators of RTA activity may indirectly modulate RTA transcriptional activity. Song et al. has shown that HMGB1 (also called HMG-1) protein, a highly conserved nonhistone chromatin protein, promotes RTA binding to different RTA target sites *in vitro* and stimulates RTA activation of target genes *in vivo*. The importance of HMGB1 in viral gene expression and replication of gammaherpesvirus was highlighted by a study using MHV-68, whose viral gene expression, as well as viral replication, was significantly reduced in HMGB1-deficient cells but can be partially restored by HMGB1 transfection (Song et al. 2004). A recent study by Wang et al showed that cellular IRF-7, an essential gene for IFN- α and - β response, competes with RTA for binding to the RTA response element and thus negatively regulates RTA activation of target genes such as ORF 57 (Wang et al. 2005). This implicates that host interferon responses, on KSHV infection, may play a role in suppressing lytic gene expression by attenuating RTA transcriptional activity by IRF-7. In addition, the stability and the DNA binding activity of the RTA protein are autoregulated through its distinct domains (Chang et al. 2005c).

5

Perspectives

After the virus enters the cell, viral gene expression control is the main determinant of viral replication, especially in the case of reactivation. Identification

of RTA as the key molecular switch for KSHV gene expression serves as the starting point for defining the viral gene expression cascade. Despite significant progress during the last decade, we are still faced with gaps in our knowledge that are critical in understanding the viral gene expression program and its underlying mechanisms. Among these issues, we would like to discuss the following five challenges.

First, we know very little about signaling upstream of RTA that controls the initiation of the lytic cycle, in comparison to the events immediately downstream of RTA. Cellular signals that positively or negatively regulate RTA expression or activity have not been systematically identified. With the available genomic approaches, this question can be addressed in the near future. It is expected that multiple cellular signaling pathways affect the balance between latency and lytic replication. The next challenge is how to define the combinatory effects of these multiple signals that simultaneously act on the infected cells. The current approaches in molecular or cellular biology usually examine one or two signaling pathways at a time. New methods must be introduced to define the biological effects of multiple inputs. This advance will help us to understand the mechanism coupling viral reactivation with cellular differentiation and associated pathogenesis (Young et al. 1991; Longworth and Laimins 2004; Johnson et al. 2005). On a related issue, we expect to see significant progress in defining the effects of virion proteins on RTA expression during *de novo* infection and the effects of chromatin structure on reactivation from latency.

Second, the activation of early genes by RTA was mostly studied with reporter assays. It requires direct evidence for the interactions between RTA or its partners with their DNA targets. The temporal relationship among the activation of the viral downstream genes has not been defined, although viral gene array data provide some hints at a low resolution. Chromosomal immunoprecipitation should facilitate the construction of gene expression cascade with a clearer molecular definition. A similar approach should be applied to cellular genes regulated by RTA.

Third, some lytic genes are differentially expressed among the KSHV-associated diseases (KS, PEL, and MCD). Such differential expression may contribute to the tissue tropism and the diverse disease outcomes of KSHV infection. We expect to see more studies addressing how different cellular and microenvironmental signals affect the expression of viral genes and how these varied expressions may alter disease pathogenesis.

Fourth, a gap in the current understanding of KSHV gene expression cascade is how late gene expression is controlled. Because some early genes and most late genes are not directly activated by RTA, the intermediator(s) functioning downstream of RTA must be identified. A current assumption

is that the late genes will be expressed once the viral DNA is replicated. How viral late gene expression is coupled to viral DNA replication is a long-standing question. Our recent study with MHV-68 suggested that additional viral factors are required in addition to the viral DNA replication *in cis*. The mechanism controlling late gene expression is a less well-defined area in the herpesvirus field.

Finally, the biological relevance of the majority of the studies reviewed here must be validated in the context of viral replication. The availability of a BAC system to manipulate the KSHV genome will greatly facilitate such work (Zhou et al. 2002; Gao et al. 2003). The next challenge is to apply the knowledge to clinical applications for both tumor therapy and prevention. Based on the concept that KSHV lytic replication plays a direct role in tumor development by providing a favorable microenvironment, inhibition of lytic gene expression may have therapeutic values. On the other hand, intentional induction of lytic gene expression in the presence of a gancyclovir-type drug may drive the destruction of tumor lesions because of the elevated immune responses and the bystander killing effect of gancyclovir.

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Kaposi Sarcoma Herpesvirus-Encoded Interferon Regulator Factors

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1	Consequences of Infection with KSHV	186
2	KSHV-Encoded Proteins with Homology to the IRF Family of Transcription Factors	187
3	Subversion of Host Antiviral Defenses by vIRFs	187
4	Alterations in IFN Induction and Responsiveness Resulting from vIRF-1 .	190
4.1	vIRF-1 Expression	192
5	Alteration in IFN Induction and Responsiveness Resulting from vIRF-2 .	193
5.1	vIRF-2 Expression	193
6	Alterations in IFN Induction and Responsiveness Resulting from vIRF-3 .	194
6.1	vIRF-3 Expression	195
7	vIRF-4 Expression	195
8	Inhibition of p53 by vIRFs	195
9	Inhibition of NF- κ B by vIRFs	196
10	Inhibition of Transforming Growth Factor- β Signaling by vIRF-1	197
11	Expression of IRF-Regulated Gene Products in KSHV-Infected Cells and Responses to Exogenous IFNs	198
12	Other KSHV-Encoded Proteins That Block Responsiveness to IFNs	199
13	Concluding Remarks	200
	References	201

Abstract The Kaposi sarcoma herpesvirus (KSHV) encodes multiple proteins that disrupt host antiviral responses, including four viral proteins that have homology to the interferon regulatory factor (IRF) family of transcription factors. At least three

of the KSHV vIRFs (vIRFs 1–3) alter responses to cellular IRFs and to interferons (IFNs), whereas functional changes resulting from the fourth vIRF (vIRF-4) have not been reported. The vIRFs also affect other important regulatory proteins in the cell, including responses to transforming growth factor β (TGF- β) and the tumor suppressor protein p53. This review examines the expression of the vIRFs during the life cycle of KSHV and the functional consequences of their expression.

1 Consequences of Infection with KSHV

Kaposi sarcoma herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a large double-stranded DNA virus belonging to the gammaherpesvirus subfamily (Boshoff and Weiss 1998; Moore and Chang 2001). As with all herpesviruses, once an individual becomes infected with KSHV, the virus persists throughout the lifetime of the host. This lifelong persistence occurs because KSHV enters a latent state that is not well recognized by innate and acquired host antiviral defenses (Moore and Chang 2003). During latency, viral replication is coupled to cellular replication without producing infectious virus (Rainbow et al. 1997; Ballestas et al. 1999; Hu et al. 2002), and viral gene products are expressed that subvert cellular antiviral defenses so that infected cells are not destroyed (Friborg et al. 1999; Brander et al. 2000; Radkov et al. 2000; Guasparri et al. 2004; An et al. 2005). Production of infectious virus can occur when KSHV shifts from latent to lytic replication (Renne et al. 1996; Lukac et al. 1998). KSHV expresses proteins during the lytic cascade that disrupt components of the cellular antiviral machinery and the host response, thereby enhancing the likelihood of successful production of infectious virus (Gao et al. 1997; Sarid et al. 1997; Zimring et al. 1998; Coscoy and Ganem 2000; Means et al. 2002; Pozharskaya et al. 2004b).

Although most individuals who are latently infected with KSHV remain asymptomatic and never experience KSHV-associated diseases, the lifelong persistence of KSHV puts the host at risk for the development of diseases associated with KSHV, including Kaposi sarcoma (KS), primary effusion lymphoma (PEL), and a plasmacytic variant of multicentric Castleman disease (Moore and Chang 2003). Tumor cells in both KS and PEL contain KSHV that is primarily latent (Parravicini et al. 2000; Dittmer 2003), but a few cells in KS lesions support lytic replication and express lytic viral proteins that contribute to the disease process (Staskus et al. 1997; Cannon et al. 2003). Many of the KSHV-infected cells in multicentric Castleman disease support lytic replication of KSHV, with viral proteins expressed during the lytic cascade playing a critical role in the disease process (Dupin et al. 1999; Parravicini et al. 2000; Waterston and Bower 2004).

2 KSHV-Encoded Proteins with Homology to the IRF Family of Transcription Factors

KSHV has significant sequence homology with other herpesviruses, with conservation of many open reading frames (ORFs) that encode proteins that are critical for herpesvirus replication and packaging of virions. KSHV ORFs were named after their homologues in herpesvirus saimiri (ORFs 1–75), whereas ORFs without recognizable homologues in herpesvirus saimiri were numbered separately as K1–K15 (Russo et al. 1996; Neipel et al. 1997). ORFs or Ks with decimals refer to ORFs that were not identified before the initial numbering (Neipel et al. 1997). Many of the proteins encoded by the Ks have homology to mammalian proteins, including four viral proteins that have homology to the interferon (IFN) regulatory factor (IRF) family of transcription factors. All four vIRFs are located between ORF57 and ORF58 on the KSHV genome (Sarid et al. 1998; Lubyova and Pitha 2000; Rivas et al. 2001; Cunningham et al. 2003). The splicing of vIRF-2 and vIRF-3 has led to several different reported locations on the KSHV genome (Table 1). vIRF-1 is located at K9, vIRF-2 at K11.1, vIRF-3, also called latency-associated nuclear antigen 2 (LANA2) at K10.5, and vIRF-4 at K10.

The location of the vIRFs and their sequence homologies suggest that they arose from capture of cellular sequences followed by gene duplication events. Each gene possesses its own promoter and polyadenylation site, and all except vIRF-1 are spliced from two exons. There may be some alternative splicing that leads to the minor differences in sizes of transcripts that have been detected, but there is no evidence of splicing between the different vIRFs (Cunningham et al. 2003). The only other herpesvirus that is known to encode vIRFs is the rhesus rhadinovirus (RRV), a close relative to KSHV (Searles et al. 1999; Alexander et al. 2000). The RRV encodes eight tandem vIRFs, but, unlike KSHV, none of them are spliced.

3 Subversion of Host Antiviral Defenses by vIRFs

Nine cellular IRF genes have been identified that function in the regulation of expression of IFN and other genes (Pitha et al. 1998), and at least three of the KSHV vIRFs (vIRFs 1–3) alter responses to cellular IRFs and to IFNs (Gao et al. 1997; Flowers et al. 1998; Zimring et al. 1998; Burysek et al. 1999a; Burysek et al. 1999b; Burysek and Pitha 2001; Lin et al. 2001; Nakamura et al. 2001; Lubyova et al. 2004) (Table 2). The functional changes resulting from

Table 1 vIRFs located between ORF 57 and ORF 58

	Reported genomic locations	Splicing pattern	Expression pattern	Cellular location
vIRF-1	K9 (Russo et al. 1996)	Not spliced (Cunningham et al. 2003)	Low constitutive expression during latency (Dittmer 2003; Pozharskaya et al. 2004b); strongly induced during lytic cascade (Pozharskaya et al. 2004b)	Predominantly in nucleus and sometimes in cytoplasm (Inagi et al. 1999; Parravicini et al. 2000; Pozharskaya et al. 2004b)
vIRF-2	K11.1 spliced with K11 (Jenner et al. 2001; Cunningham et al. 2003)	Spliced (Cunningham et al. 2003)	Cell type variation; some expression during latency (Burysek et al. 1999b; Burysek and Pitha 2001; Cunningham et al. 2003); sometimes strongly induced during lytic cascade (Cunningham et al. 2003)	GFP-vIRF-2 fusion protein expressed in nucleus (Burysek and Pitha 2001)
vIRF-3/ LANA 2	K10.1, K10.5, K10.6 (Lubyova and Pitha 2000; Rivas et al. 2001)	Spliced (Lubyova and Pitha 2000; Rivas et al. 2001; Cunningham et al. 2003)	Constitutively expressed during latency (Rivas et al. 2001; Fakhari and Dittmer 2002); some increase at the mRNA but not protein level in response to TPA (Lubyova and Pitha 2000; Lubyova et al. 2004)	Nucleus (Rivas et al. 2001; Munoz-Fontela et al. 2003)
vIRF-4	K10	Spliced (Cunningham et al. 2003)	Strongly induced during lytic cascade (Cunningham et al. 2003)	Not reported

vIRF-4 have not been reported. The cellular IRFs play a critical role in the response to viral infection (Pitha et al. 1998; Barnes et al. 2002). All of the IRFs share homology in the amino-terminal region, including five tryptophan repeats that regulate binding to DNA (Taniguchi et al. 2001; Barnes et al. 2002). Some of the IRFs serve as transcriptional activators, whereas others serve as transcriptional repressors. The vIRFs do not retain the amino acids that are utilized by the nine known cellular IRFs for binding to DNA. It is thus not

Table 2 Changes in gene expression and protein activation resulting from vIRFs

	vIRF-1	vIRF-2	vIRF-3
Virus-induced type I IFN expression	Inhibited (Lin et al. 2001)	Inhibited (Burysek et al. 1999b)	Inhibited in L929 cells (Lubyova and Pitha 2000); Induced in BJAB cells (Lubyova et al. 2004)
Gene expression induced by IFN α , β , or γ	Inhibited (Flowers et al. 1998; Li et al. 1998; Zimring et al. 1998)	Not reported	Not reported
Activation of IFN-induced proteins	Not reported	Activation of PKR blocked (Burysek and Pitha 2001)	Activation of PKR blocked, whereas activation of 2',5'-OAS not affected (Esteban et al. 2003)
NF- κ B-induced gene expression	Not inhibited (Zimring et al. 1998)	Inhibited (Burysek et al. 1999b)	Inhibited (Seo et al. 2004)
p53-induced apoptosis and gene expression	Inhibited (Seo et al. 2001)	Not reported	Inhibited (Rivas et al. 2001)
IRF-1-induced gene expression	Inhibited (Flowers et al. 1998; Zimring et al. 1998)	Inhibited (Burysek et al. 1999b)	Not reported
IRF-3-induced gene expression	Inhibited (Lin et al. 2001)	Inhibited (Burysek et al. 1999b)	Inhibited in L929 cells (Lubyova and Pitha 2000); induced in BJAB cells (Lubyova et al. 2004)
IRF-7-induced gene expression	Not inhibited (Lin et al. 2001)	Not reported	Inhibited in L929 cell (Lubyova and Pitha 2000); induced in BJAB cells (Lubyova et al. 2004)

surprising that none of the vIRFs binds to the sites in DNA to which the cellular IRFs bind (Flowers et al. 1998; Zimring et al. 1998; Lin et al. 2001). They also do not prevent the cellular IRFs from binding to DNA. They nonetheless can disrupt the function of multiple cellular IRFs.

Most studies on the functions of vIRFs have used expression vectors to constitutively express vIRFs in non-KSHV-infected cells, thereby characterizing the consequence of vIRF expression in the absence of other viral proteins. In addition, studies have been done in KSHV-infected PEL cells (Li et al.

1998, 2000; Nakamura et al. 2001; Zhou et al. 2002; Pozharskaya et al. 2004b). These studies demonstrate that vIRFs affect multiple antiviral pathways, with some shared and some distinct functions occurring in response to the different vIRFs.

All three vIRFs that have been characterized alter IFN expression and/or signaling. IFNs play an important role in the host response to viral infection (Samuel 2001; Malmgaard 2004). The type I IFNs consist of multiple forms of IFN α and a single IFN β , whereas type II IFN consists exclusively of IFN γ (Malmgaard 2004). There are distinct receptors for type I and type II IFNs, but they share some components of the Jak-Stat signaling pathway and induce expression of some shared genes, including proteins that play an important role in cellular and host defenses against viral infection (Kotenko and Pestka 2000; Pozharskaya et al. 2004a). Proteins such as the double-stranded RNA activated protein kinase (PKR) and 2',5'-oligoadenylate synthetase (2'5'-OAS) that are induced by both types of IFN display very little enzymatic activity unless they are activated, a process that often occurs in response to molecular motifs encountered during viral infection (Rebouillat and Hovanessian 1999; Justesen et al. 2000; Vattem et al. 2001; Chawla-Sarkar et al. 2003; Donze et al. 2004). The activated enzymes then inhibit protein synthesis, thereby impairing the ability of viruses to survive and replicate.

For viruses to persist within the host, the antiviral effects of IFNs generally must be subverted. vIRF-1 and vIRF-2 block transcription of type I IFN, whereas vIRF-3 can either block or enhance transcription of type I IFN, depending on cellular context (Burysek et al. 1999a, 1999b; Li et al. 2000; Lubyova and Pitha 2000; Lin et al. 2001; Lubyova et al. 2004). Both vIRF-2 and vIRF-3 block the activation of the IFN-inducible antiviral protein PKR, and they also block activation of NF- κ B (Burysek et al. 1999b; Esteban et al. 2003; Seo et al. 2004). In addition, vIRF-1 and vIRF-3 bind to the tumor suppressor protein p53, blocking p53-induced transcription and apoptosis (Nakamura et al. 2001; Rivas et al. 2001; Seo et al. 2001; Munoz-Fontela et al. 2003). Thus the vIRFs affect the function of multiple important regulatory pathways in a way that generally favors the survival of virally infected cells.

4

Alterations in IFN Induction and Responsiveness Resulting from vIRF-1

The expression of vIRF-1 blocks transcription of type I IFNs as well as responses induced by IFN α , IFN β , and IFN γ (Li et al. 1998; Zimring et al. 1998; Lin et al. 2001). The inhibition results in large part from the binding of vIRF-1 to the coactivator proteins CBP and p300, thereby interfering with coactivator

binding and function (Burysek et al. 1999a; Li et al. 2000; Seo et al. 2000; Lin et al. 2001). CBP and p300 are structurally and functionally related proteins that possess histone acetyltransferase (HAT) activity, and they associate with CBP/p300-associated factor (pCAF), another protein that has HAT activity (Chan and La Thangue 2001). The interaction between vIRF-1 and p300 displaces p/CAF from p300, thereby inhibiting the HAT activity of p300 (Li et al. 2000). In addition, vIRF-1 can displace CBP/p300 from IRF-3, thereby inhibiting transcriptional activation by IRF-3 (Lin et al. 2001). HAT activity is important for altering chromatin structure and making the DNA accessible for transcription (Chan and La Thangue 2001). The disruption of CBP/p300 binding and function inhibits transcriptional activation by IRF-3, whereas transcriptional activation by IRF-7 is less affected (Suhara et al. 2000; Lin et al. 2001; Yang et al. 2002, 2003, 2004).

Both cellular IRF-3 and IRF-7 reside in the cytoplasm, and activation requires phosphorylation followed by nuclear translocation. Activated IRF-3 binds to CBP/p300 in order to become transcriptionally active (Suhara et al. 2000; Yang et al. 2002). IRF-3 and IRF-7 continue to undergo phosphorylation and bind to DNA when vIRF-1 is expressed, but IRF-3 is no longer able to enhance transcription (Lin et al. 2001). Because IRF-3 is required for the transcription of IFN α and IFN β , the disruption of IRF-3 function that results from vIRF-1 blocks expression of all forms of type I IFN (Lin et al. 2001). The IRF-7 transcription factor contributes to type I IFN expression (Yang et al. 2003, 2004). vIRF-1 binds to cellular IRF-7, but IRF-7 continues to enhance transcription because it is less dependent on CBP/p300 than IRF-3 (Lin et al. 1998, 2000a, 2000b, 2001; Li et al. 2000; Seo et al. 2000; Morin et al. 2002; Servant et al. 2002; Sharma et al. 2003).

The expression of vIRF-1 also blocks the ability of IRF-1 to enhance transcription (Zimring et al. 1998; Burysek et al. 1999a). IRF-1 is generally induced to higher levels of expression by both type I and type II IFN, and it serves as a transcription factor for many cellular proteins involved in regulating proliferation and immune responses (Coccia et al. 1999; Taniguchi et al. 2001; Kroger et al. 2002; Romeo et al. 2002; Lee et al. 2003; Dornan et al. 2004). IRF-1 also has activities that complement the activities of other transcription factors such as p53. For example, IRF-1 is important in the acetylation of p53 through its recruitment of p300 to the p21 promoter, and this enhances transcriptional activation by p53 (Dornan et al. 2004). Thus the disruption of IRF-1 function affects the expression of many cellular genes.

Because vIRF-1 blocks the transcriptional activity of several IRF family members, it has profound effects on induction of IFN-regulated, immunoregulatory and growth regulatory gene products. Microarray analysis of cells expressing vIRF-1 demonstrate that approximately 7% of the 4,500 transcripts

examined changed by at least twofold (Li et al. 2000). The expression of vIRF-1 inhibits apoptosis (Burysek et al. 1999a; Seo et al. 2001) and induces malignant transformation of several different cell types (Gao et al. 1997; Li et al. 1998).

The inhibition of HAT activity that results from vIRF-1 causes some global changes in the chromatin. Cells expressing vIRF-1 have reduced histone acetylation and condensation of the chromatin, thereby reducing the ability of propidium iodide to bind stoichiometrically to DNA (Li et al. 2000). This can lead to falsely low quantitation of cellular DNA content, whereas binding of propidium iodide to DNA can be restored to normal levels by incubation with the histone deacetylase inhibitor butyrate.

4.1

vIRF-1 Expression

Studies in the primary effusion lymphoma cell line BCBL-1 demonstrate that vIRF-1 is expressed during both latent and lytic replication (Pozharskaya et al. 2004b) (Table 1). The levels of vIRF-1 that are achieved during lytic replication are much higher than during latency, and hence the latent expression is often overlooked (Parravicini et al. 2000; Dittmer 2003). The amount of vIRF-1 that is expressed during latency is insufficient to block responses to exogenous IFNs in BCBL-1 cells, whereas the high levels expressed during lytic replication block gene induction by exogenous IFN α (Pozharskaya et al. 2004b). The vIRF-1 expressed during latency is associated with PML bodies, but the functional consequence of this association is not known. When vIRF-1 is expressed at the high levels that occur during lytic replication of KSHV, it is often detected in the cytoplasm as well as in the nucleus (Inagi et al. 1999; Parravicini et al. 2000; Pozharskaya et al. 2004b). Immunoprecipitation studies reveal that 5%–10% of vIRF-1 in TPA-stimulated BCBL-1 cells is bound to p300 (Li et al. 2000), providing evidence that much of the vIRF-1 that is expressed during the course of KSHV infection is complexed with p300. When KSHV in BCBL-1 cells is induced to enter the lytic cascade, the percentage of cells that express high levels of vIRF-1 is considerably lower than the percentage that express other lytic markers, such as the product of ORF 59 (Pozharskaya et al. 2004b). This is because vIRF-1 is transiently expressed and has a short half-life, whereas some other lytic viral proteins are more stable. Thus vIRF-1 is able to inhibit cellular antiviral defenses for only a limited portion of the lytic cascade.

The level of expression of vIRF-1 in latently infected cells is beneath the threshold for detection unless a method that is more sensitive than immunohistochemistry is used. vIRF-1 protein is readily detectible in multifocal Castleman disease and in cultured PEL cells supporting lytic replication, whereas vIRF-1 is not detected by immunohistochemical analysis in freshly

excised KS biopsies or in primary effusion lymphomas (Parravicini et al. 2000). It is nonetheless expressed in latently infected KS biopsies as revealed by microarray analysis, and its expression profile in KS biopsies corresponds with latent rather than lytic expression (Dittmer 2003).

vIRF-1 is transcribed in both uninduced and induced cells from a single initiating site preceded by a TATA box (Chen et al. 2000; Cunningham et al. 2003). An additional TATA box and initiation site is used to initiate vIRF-1 transcription during latency. vIRF-1 transcription is induced by replication and transcription activator (Rta), the viral gene product that induces lytic replication of KSHV (Ueda et al. 2002). vIRF-1 has a transactivation domain that can drive transcription (Roan et al. 1999), and vIRF-1 autoactivates its own expression (Wang and Gao 2003).

5

Alteration in IFN Induction and Responsiveness Resulting from vIRF-2

In transient transfection assays, the expression of vIRF-2 disrupts the ability of viruses to induce type I IFNs (Burysek et al. 1999b) (Table 2). vIRF-2 inhibits NF- κ B and IRF-1-dependent transcription (Burysek et al. 1999b). This blocks IFN β transcription because the coordinate activation of both IRF family members and NF- κ B are required for IFN β transcription (Parekh and Maniatis 1999; Han et al. 2004).

The expression of vIRF-2 blocks the activation of PKR (Burysek and Pitha 2001), an antiviral protein that is induced by IFN and activated by molecular motifs such as double-stranded RNA that are encountered during viral infection (Clemens 1997; Tan and Katze 1999; Justesen et al. 2000). PKR catalyzes the phosphorylation of eIF2 α , a translation factor that blocks the initiation of protein synthesis when it becomes phosphorylated (Clemens and Elia 1997; Samuel et al. 1997). The prevention of activation of PKR by vIRF-2 decreases the ability of cells to respond to viral infection with reduced protein synthesis as part of their antiviral strategy. Thus vIRF-2 not only blocks expression of IFN β but also blocks the functional consequences of IFNs by blocking the activation of an IFN-induced protein.

5.1

vIRF-2 Expression

Initial studies classified vIRF-2 as a latent viral gene because its mRNA and protein levels did not change in response to agents that induce lytic replication (Burysek et al. 1999b; Burysek and Pitha 2001) (Table 1). Other investigators

have reported that ν IRF-2 levels increase in response to TPA, thereby reclassifying it as a lytic transcript (Cunningham et al. 2003). When different types of KSHV-infected cells were examined for ν IRF-2 mRNA expression with reverse transcriptase-PCR, some KSHV-infected cells showed considerable expression of ν IRF-2 in unstimulated cells, whereas other cell lines showed minimal expression of ν IRF-2 before induction of lytic replication with TPA (Cunningham et al. 2003). This suggests that ν IRF-2 might be expressed during latent and lytic replication of KSHV, with some cell type differences. Expression of green fluorescent protein-tagged ν IRF-2 in non-KSHV-infected cells provides evidence that ν IRF-2 is a nuclear protein (Burysek and Pitha 2001). The percentage of KSHV-infected cells that express ν IRF-2 is not known, and viral proteins that are coexpressed with ν IRF-2 have not been reported.

6

Alterations in IFN Induction and Responsiveness Resulting from ν IRF-3

Some studies report that IFN α transcription and expression increase in response to ν IRF-3 (Lubyova et al. 2004), whereas other studies show ν IRF-3 inhibits IFN α expression (Lubyova and Pitha 2000) (Table 2). When L929 cells were transfected with an expression vector for ν IRF-3, it decreased virus-induced transcriptional activation of an IFN α -reporter construct and decreased the amount of biologically active IFN α that was produced in response to Newcastle disease virus (NDV) infection (Lubyova and Pitha 2000). In contrast, ν IRF-3 increased virus-induced transcription and production of IFN α in BJAB cells (Lubyova et al. 2004). ν IRF-3 binds to activated cellular IRF-3, whereas ν IRF-3 binds to cellular IRF-7 irrespective of its activation state (Lubyova et al. 2004). ν IRF-3 binds to a site in p300 that is distinct from the site that binds to ν IRF-1, and the interaction between ν IRF-3 and CBP/p300 enhances HAT activity. The transcriptional complexes that contain ν IRF-3 are recruited to the IFN α promoter and more effectively enhance transcription than in the absence of ν IRF-3. This increases the amount of IFN α produced by BJAB cells infected with NDV by approximately fourfold compared to control cells lacking ν IRF-3 (Lubyova et al. 2004). The increase in IFN production that occurs when ν IRF-3 is expressed in BJAB cells differs from the reduction that occurs when ν IRF-3 is expressed in L929 cells (Lubyova and Pitha 2000), suggesting there might be cell type differences in the functional consequences of ν IRF-3 expression.

6.1

vIRF-3 Expression

Initial studies suggested that vIRF-3 was expressed during lytic replication because mRNA levels increased when BCBL-1 cells were incubated with TPA (Lubyova and Pitha 2000), but vIRF-3 protein levels do not increase when BCBL-1 cells are incubated with TPA (Lubyova et al. 2004). Expression of vIRF-3 is detected at the protein level in the nuclei of all latently infected BCBL-1 cells with a speckled pattern that is distinct from the pattern that results from LANA (Rivas et al. 2001) (Table 2). Its constitutive expression during latency is responsible for vIRF-3 having an alternative name, LANA2 (Rivas et al. 2001; Esteban et al. 2003; Munoz-Fontela et al. 2003). vIRF-3 is detected in nearly all PEL tumor cells and the many KSHV-associated Castleman disease cells, but vIRF-3 is not expressed in KS tumors (Rivas et al. 2001; Fakhari and Dittmer 2002, 2003). Thus there are tissue-specific differences in the expression of vIRF-3, with expression occurring in cells of B lymphocyte origin that are latently infected with KSHV but not in cells of endothelial origin. vIRF-3 is encoded by a spliced mRNA and can exist in two different sizes, 2.2 or 1.8 kb (Lubyova and Pitha 2000; Rivas et al. 2001). The spliced transcript for vIRF-3 originates at ORFK10.5 and is composed of a 455-bp 5' exon that is joined to the 1,339-bp 3' exon previously called K10.1. The promoter of vIRF-3 is the only member of the vIRF family that lacks an obvious TATA box (Cunningham et al. 2003).

7

vIRF-4 Expression

Functional studies on vIRF-4 have not been reported. vIRF-4 is a spliced transcript that is induced by TPA in multiple KSHV-infected cell types (Sarid et al. 1998; Jenner et al. 2001; Fakhari and Dittmer 2002; Cunningham et al. 2003).

8

Inhibition of p53 by vIRFs

Expression of the tumor suppressor gene p53 is one of the mechanisms used by cells to prevent the survival and replication of virally infected cells (Collot-Teixeira et al. 2004; Meek 2004; Michalak et al. 2005; O'Shea 2005). p53 is a transcription factor that induces expression of cellular proteins that prevent replication of cells containing abnormal DNA by inhibiting proliferation

and/or inducing apoptosis (Oren 2003; Meek 2004; Slee et al. 2004; Harris and Levine 2005; Michalak et al. 2005; Sengupta and Harris 2005). Viruses often express proteins that disrupt the action of p53 in order to overcome its growth-suppressive and proapoptotic actions (Friborg et al. 1999; Moore and Chang 2003; Collot-Teixeira et al. 2004). Binding of vIRF-1 to p53 suppresses phosphorylation and acetylation of p53 and inhibits the transcriptional activation and apoptosis driven by p53 (Nakamura et al. 2001; Seo et al. 2001) (Table 2). vIRF-3 also binds to p53 and blocks its ability to enhance transcription and induce apoptosis (Nakamura et al. 2001; Rivas et al. 2001; Seo et al. 2001; Munoz-Fontela et al. 2003). Thus both vIRF-1 and vIRF-3 join other KSHV-encoded proteins such as LANA and K-bZIP that repress p53 activity (Friborg et al. 1999; Park et al. 2000). Immunofluorescent microscopy demonstrates that vIRF-1 and p53 colocalize in some KSHV-infected BCBL-1 cells, but sometimes the amount of p53 exceeds the amount that is bound by vIRF-1 (Nakamura et al. 2001; Pozharskaya et al. 2004a). Immunoprecipitation studies provide additional evidence that vIRF-1 and p53 interact in KSHV-infected cells (Nakamura et al. 2001). In contrast, vIRF-3 does not coprecipitate with p53 in KSHV infected cells, and thus it might not associate with p53 when other viral proteins are present (Rivas et al. 2001). It is not known whether the other KSHV-encoded proteins that associate with p53 compete for binding or coassociate with p53, and the relative importance of specific KSHV proteins in disrupting p53 function is not known.

9

Inhibition of NF- κ B by vIRFs

The transcription factor NF- κ B plays an important role in the cellular response to viral infection (Offermann et al. 1995; Alexopoulou et al. 2001; Harcourt and Offermann 2001; Kaiser and Offermann 2005), and it is also involved in controlling the balance between latent and lytic replication (Brown et al. 2003). NF- κ B is often activated as part of the cellular antiviral response, leading to expression of proinflammatory proteins that can enhance the host response to viral infection (Offermann et al. 1995; Alexopoulou et al. 2001; Harcourt and Offermann 2001; Kaiser et al. 2004). The activation of NF- κ B by viral proteins can also be beneficial to the virus by enhancing expression of antiapoptotic proteins that help virally infected cells escape cellular defenses (Stehlik et al. 1998; Kreuz et al. 2001; Li et al. 2001; Mak and Yeh 2002). Both vIRF-2 and vIRF-3 decrease activation of NF- κ B (Burysek et al. 1999b; Seo et al. 2004) (Table 2). vIRF-2 binds to the κ B consensus element in DNA, blocking the ability of NF- κ B to enhance transcription (Burysek et al. 1999b).

vIRF-3 inhibits the kinase activity of I κ B kinase β (IKK β) but not of IKK α (Seo et al. 2004). This blocks the nuclear translocation of NF- κ B and sensitizes cells to TNF-induced apoptosis.

The functional consequences of the alterations in NF- κ B that occur in response to vIRF-2 and vIRF-3 are unclear. The studies showing an interaction between vIRF-3 and IKK β were done with transfection of expression vectors. Immunoprecipitation studies using extracts from BCBL-1 cells did not show evidence of vIRF-3 and IKK β interactions (Seo et al. 2004), suggesting that the interaction might not occur when other viral proteins that interact with IKK β are present. NF- κ B is activated in latently infected PEL cells despite expression of vIRF-3, and inhibition of NF- κ B activation leads to apoptosis of PEL cells (Keller et al. 2000). This indicates that vIRF-3 is insufficient to inhibit NF- κ B activation in KSHV-infected BCBL-1 cells, but it might be modulating the level of activation or the function of the NF- κ B that results. The activation of NF- κ B that occurs during latency is likely to result from vFLIP, a viral protein expressed during latency that activates NF- κ B through interactions with multiple components of the IKK (Matta et al. 2003). The activated NF- κ B not only plays an important role in the survival of KSHV-infected cells (Keller et al. 2000; Belanger et al. 2001; Ghosh et al. 2003) but also helps maintain latency by inhibiting the function of Rta (Roan et al. 2002; Brown et al. 2003).

10

Inhibition of Transforming Growth Factor- β Signaling by vIRF-1

vIRF-1 also inhibits transforming growth factor- β (TGF- β) signaling, thereby suppressing the ability of TGF- β to inhibit proliferation (Seo et al. 2005). TGF- β initiates signaling by assembling a receptor kinase that activates members of the Smad family of transcription factors (Shen et al. 1998; Seoane et al. 2001; Feng and Derynck 2005; Massague et al. 2005). The activated Smad proteins reduce proliferation by inducing transcription of inhibitors of cyclin-dependent kinases, including p15 and p21, and by reducing the transcription of c-myc (Seoane et al. 2001; Massague et al. 2005). vIRF-1 binds to both Smad3 and Smad4, thereby interfering with their ability to bind to each other and to bind DNA, and this disrupts the transcriptional responses that are normally induced by TGF- β (Seo et al. 2005). Because TGF- β plays an important role in growth regulation and differentiation, its disruption by vIRF-1 is likely to further contribute to the deregulation of growth control that occurs in KSHV-infected cells.

11

Expression of IRF-Regulated Gene Products in KSHV-Infected Cells and Responses to Exogenous IFNs

When microvascular endothelial cells (MECs) are infected with KSHV, they express elevated levels of PKR and 2'5'-OAS (Krug et al. 2004). Transcription of these genes can be induced by IRF family members or by IFN (Wang and Floyd-Smith 1998; Coccia et al. 1999; Floyd-Smith et al. 1999; Yu et al. 1999; Nakaya et al. 2001). The expression of PKR is higher in MECs that are latently infected with KSHV than in surrounding uninfected cells, providing evidence that gene products expressed in virally infected cells are responsible for the increase rather than secreted IFNs (Krug et al. 2004). The increased expression of the PKR and 2'5'-OAS suggests that IRF transcription factors are activated in KSHV-infected MECs, leading to the enhanced expression of these gene products. This provides evidence that the vIRFs are not sufficient to fully block the ability of IRF family members to induce transcription of cellular antiviral gene products. The PKR and 2'5'-OAS are probably not fully activated because sufficient protein synthesis occurs in latently infected MECs for replication to continue, so that the latently infected cells have a growth advantage over uninfected cells (Krug et al. 2004). vIRF-3 is not expressed in KSHV-infected MECs (Cunningham et al. 2003), indicating that expression of vIRF-3 is not responsible for the induction of these genes or for the disruption of PKR activation.

Both type I and type II IFNs induce enhanced gene expression in KSHV-infected cells despite expression of viral proteins that disrupt responses to IRFs and to IFNs (Krug et al. 2004; Pozharskaya et al. 2004a, 2004b). Incubation of KSHV-infected MECs with IFN α for 24 h enhances expression of PKR in latently infected cells without reducing cell number, demonstrating that KSHV-infected cells respond to IFN α with increased PKR expression yet cells continue to survive (Krug et al. 2004). BCBL-1 cells that are latently infected with KSHV respond to IFN α and IFN γ with increased expression of the antiviral proteins PKR and 2'5'-OAS, yet very little cell death is observed unless KSHV enters the lytic phase of viral replication (Pozharskaya et al. 2004a, 2004b). This suggests that either these antiviral proteins are not activated or viral proteins expressed during latency block the functional consequences of activated PKR and 2'5'-OAS. Both vIRF-2 and vIRF-3 can block the activation of PKR (Burysek and Pitha 2001; Esteban et al. 2003), but vIRF-3 does not prevent activation of 2'5'-OAS (Esteban et al. 2003), and vIRF-3 is not expressed in KSHV-infected MECs (Cunningham et al. 2003). The vFLIP that is expressed during latency blocks apoptosis (Sturzl et al. 1999; Belanger et al. 2001; Sun et al. 2003; Guasparri et al. 2004), and this probably inhibits the apoptosis

that occurs when protein synthesis decreases in response to activated PKR and 2'5'-OAS (Justesen et al. 2000; Ghosh et al. 2001; Vorburger et al. 2002; Chawla-Sarkar et al. 2003; Donze et al. 2004).

The host response to viral infection is generally greater during lytic replication when multiple viral gene products are expressed, increasing the targets that can be recognized by innate antiviral defenses (Samuel 2001; Chawla-Sarkar et al. 2003; Malmgaard 2004). It is thus not surprising that most of the vIRFs and other viral gene products that block components of the cellular antiviral response are expressed during lytic replication. Apoptosis occurs in most BCBL-1 cells supporting lytic replication of KSHV (D'Agostino et al. 1999), but it generally does not prevent the release of infectious virus unless the process is accelerated by incubation with either IFN α or IFN γ (D'Agostino et al. 1999; Pozharskaya et al. 2004a, 2004b; Klass et al. 2005). The apoptosis that occurs during lytic replication is not dependent on viral DNA replication or viral late gene expression because it continues to occur when virus production is blocked with either ganciclovir or phosphoformic acid (Klass et al. 2005). Thus the role of expression of vIRFs and other viral gene products that disrupt the innate antiviral response may be to delay and/or attenuate the cellular response until production of infectious virus is complete, but they are not sufficient to allow production of infectious virus when the host response is augmented by exogenous IFN encountered early in the lytic cascade (Pozharskaya et al. 2004b).

The majority of the cells that are in the lytic cascade do not express high levels of vIRF-1 because vIRF-1 transcription is transiently induced and vIRF-1 protein has a short half-life (Pozharskaya et al. 2004b). The percentage of cells within the lytic cascade that express vIRF-2 or vIRF-4 is not known, and the duration of their expression during the lytic cascade is also not known. When cells supporting lytic replication are incubated with either IFN α or IFN γ , increased expression of PKR, 2'5'-OAS, and IRF-1 occurs, and the production of infectious virus is reduced, especially when the IFNs are added early in the lytic cascade (Pozharskaya et al. 2004a, 2004b). Thus none of the vIRFs is sufficient to fully protect KSHV-infected MECs from the antiviral effects of IFN α and IFN γ .

12

Other KSHV-Encoded Proteins That Block Responsiveness to IFNs

A structurally unrelated KSHV protein encoded by ORF45 binds cellular IRF-7 and blocks its phosphorylation and nuclear translocation, thereby blocking the induction of IFN α during KSHV infection (Zhu et al. 2002). The disrupt-

tion of IRF-7 function should also reduce expression of IFN β (Yang et al. 2003). The ORF45 protein is expressed early in the lytic cascade, and the expression persists throughout the lytic cascade so that ORF45 protein is found in KSHV virions as a tegument protein (Zhu and Yuan 2003). Unlike the vIRFs that are expressed in a subset of gammaherpesviruses, ORF45 is conserved among members of the gammaherpesvirus subfamily (Jia et al. 2005). A murine gammaherpesvirus 68 (MHV68) mutant lacking ORF45 loses infectivity, whereas it is restored by expression of KSHV ORF45. This provides evidence that disruption of IRF-7 function and type I IFN expression must occur for herpesviruses to infect and replicate in mammalian cells. ORF45 is expressed early and throughout the lytic cascade (Zhu et al. 2002; Zhu and Yuan 2003), yet virally infected cells supporting lytic replication continue to respond to IFNs (Monini et al. 1999; Krug et al. 2004; Pozharskaya et al. 2004a, 2004b). Thus the vIRFs and ORF45 are unable to block responses to high levels of exogenous IFN, suggesting that their role is to disrupt responses to endogenous IRFs, IFNs, and other components of the cellular antiviral response, thereby enhancing production of infectious virus.

13

Concluding Remarks

It is likely that the vIRFs are not essential for viral replication because their incorporation into herpesviruses is relatively recent and is not found in the majority of gammaherpesviruses. They nonetheless are likely to provide some protection from innate and acquired antiviral defenses. Each of the characterized vIRFs affects the transcription of type I IFNs, and they also affect responses induced by IFNs. vIRFs also block activation of PKR, activation of p53, and activation of the NF- κ B pathway. They alter the expression of a wide range of cellular and viral gene products by altering the responses to multiple transcription factors including IRF-1, IRF-3, IRF-7, p53, NF- κ B, and Smads. Several vIRFs block apoptosis, and at least one of the vIRFs can induce malignant transformation. Thus their expression is likely to have a significant impact on the phenotype of KSHV-infected cells.

Very little is known about the function of the vIRFs in the context of KSHV infection. KSHV with deletion of vIRF-1 maintains its ability to infect cells and produce infectious virus (Zhou et al. 2002), but it is not known whether the deletion of vIRF-1 alters the amount of infectious virus that is produced, the cellular response to the virus, or the ability of IFNs to modulate the cellular response. There are no reports on the consequences of deletion of the other vIRFs. vIRF-3 is constitutively expressed during latent infection with

KSHV, but its expression is limited to cells of B lymphocyte origin and hence is not essential for protecting latently infected cells from cellular defenses. Expression of both vIRF-2 and vIRF-4 increases in response to agents that induce lytic replication, but it is not known when in the lytic cascade these proteins are expressed and whether they are expressed at sufficient levels to modulate the processes that are affected when they are overexpressed by transfection. There are no reports on the functional consequences of vIRF-4 expression, and no studies have been reported using KSHV that is defective in the expression of vIRF-2, vIRF-3, or vIRF-4. The functional consequence of the vIRF-1 that colocalizes with PML bodies in all KSHV-infected cells is currently unknown. Thus future studies will need to explore the consequences of de novo infection using KSHV with targeted deletion of the vIRFs compared to wild-type virus, with special attention to pathways that have been shown to be affected by transfected vIRFs.

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Endothelial Cell- and Lymphocyte-Based In Vitro Systems for Understanding KSHV Biology

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1	Description and Classification of Kaposi Sarcoma	212
2	Kaposi Sarcoma-Associated Herpesvirus	213
3	KSHV-Infected Cell Lines Derived from Primary Effusion Lymphomas . .	216
4	Experimental Infection of Endothelial Cells with KSHV	218
5	Parental Lineage of Spindle Cells	229
6	A Model of KS Lesion Progression	231
	References	233

Abstract Kaposi sarcoma (KS), the most common AIDS-associated malignancy, is a multifocal tumor characterized by deregulated angiogenesis, proliferation of spindle cells, and extravasation of inflammatory cells and erythrocytes. Kaposi sarcoma-associated herpesvirus (KSHV; also human herpesvirus-8) is implicated in all clinical forms of KS. Endothelial cells (EC) harbor the KSHV genome *in vivo*, are permissive for virus infection *in vitro*, and are thought to be the precursors of KS spindle cells. Spindle cells are rare in early patch-stage KS lesions but become the predominant cell type in later plaque- and nodular-stage lesions. Alterations in endothelial/spindle cell physiology that promote proliferation and survival are thus thought to be important in disease progression and may represent potential therapeutic targets. KSHV encodes genes that stimulate cellular proliferation and migration, prevent apoptosis, and counter the host immune response. The combined effect of these genes is thought to drive the proliferation and survival of infected spindle cells and influence the lesional microenvironment. Large-scale gene expression analyses have revealed that KSHV infection also induces dramatic reprogramming of the EC transcriptome. These changes in cellular gene expression likely contribute to the development of the KS lesion. In addition to KS, KSHV is also present in B cell neoplasias including primary effusion lymphoma and multicentric Castleman disease. A combination of virus and virus-induced host factors are similarly thought to contribute to establishment and progression of these malignancies. A number of lymphocyte- and EC-based systems

have been developed that afford some insight into the means by which KSHV contributes to malignant transformation of host cells. Whereas KSHV is well maintained in PEL cells cultured in vitro, explanted spindle cells rapidly lose the viral episome. Thus, endothelial cell-based systems for studying KSHV gene expression and function, as well as the effect of infection on host cell physiology, have required in vitro infection of primary or life-extended EC. This chapter includes a review of these in vitro cell culture systems, acknowledging their strengths and weaknesses and putting into perspective how each has contributed to our understanding of the complex KS lesional environment. In addition, we present a model of KS lesion progression based on findings culled from these models as well as recent clinical advances in KS chemotherapy. Thus this unifying model describes our current understanding of KS pathogenesis by drawing together multiple theories of KS progression that by themselves cannot account for the complexities of tumor development.

1

Description and Classification of Kaposi Sarcoma

Moritz Kaposi, a dermatologist in Vienna, described the case histories of five middle-aged and elderly men who presented with “multiple idiopathic pigmented sarcomas of the skin” in 1872 (Kaposi 1872); this disease was named Kaposi sarcoma (KS) a few years later, and remained a medical rarity in industrialized countries for over a hundred years. In 1981, an aggressive variant of KS became one of the sentinel diseases of the AIDS pandemic (Friedman-Kien et al. 1982). The poor prognosis of AIDS-associated KS led one playwright to describe the disease as “the wine-dark kiss of the angel of death” (Kushner 1995). Four distinct epidemiological forms of KS are now recognized (Dourmishev et al. 2003) and are distinguished by relative severity, HIV serostatus, and geographic distribution: classic, the variant first described in elderly Mediterranean men; AIDS-KS, severe and often fatal, the most common AIDS-associated neoplasm; endemic, an aggressive but non-HIV-associated variant common in sub-Saharan Africa; and iatrogenic, a form occasionally complicating organ transplantation. In all forms, KS usually presents as multiple reddish-purple dermal or mucosal lesions that may be flat or raised. Lesions may coalesce as disease progresses, becoming nodular and even ulcerated. Dissemination to visceral organs, including the lungs, liver, lymph nodes, and gut may also occur and is associated with poor prognosis. The four epidemiological forms of KS are histologically indistinguishable, characterized by disorganized networks of abnormal microvasculature composed of spindle-shaped cells (Flaitz et al. 1996); these cells do not maintain the integrity of microvascular channels, accounting for lesional edema and abundant extravasation of inflammatory cells and erythrocytes. Spindle cells,

thought to be of endothelial lineage, are present in early patch-stage lesions but become the predominant cell type in later plaque- and nodular-stage lesions (Aluigi et al. 1996; Beckstead et al. 1985; Regezi et al. 1993, 1993b; Roth et al. 1992, 1988; Rutgers et al. 1986; Scully et al. 1988). Patch-stage lesions are typically polyclonal, but oligoclonal and monoclonal later-stage lesions have been described (Gill et al. 1998; Judde et al. 2000; Rabkin et al. 1997). Thus, KS exhibits characteristics of both a reactive hyperproliferative lesion and a truly transformed sarcoma.

2 Kaposi Sarcoma-Associated Herpesvirus

Representational difference analysis (RDA) is a technique used to identify minor differences between complex genomic samples. DNA from KS tissue and normal skin was compared by RDA, leading to the discovery of Kaposi sarcoma-associated herpesvirus (KSHV) in 1994 (Chang et al., 1994). Sequences from this new human herpesvirus were detected in nearly all KS tumors but not in most other pathologic samples examined. Importantly, viral sequences were found in lymph node biopsies from gay men with AIDS, which suggested a link not only to patients with KS but also to groups known to be at increased risk of developing KS. Seroprevalence studies have since shown that KSHV infection is more common in populations known to be at greater risk for developing KS (Herndier and Ganem 2001) and that KSHV viral load increases before the onset of disease [although the utility of viral load as a predictor of KS development is debated (Engels et al. 2003; Polstra et al. 2004; Serraino et al. 2001)]. KSHV is now considered necessary for all clinical forms of KS (Boshoff et al. 1995; Chang et al. 1994; Cheung 2004a) as well as two rare lymphoproliferative disorders, primary effusion lymphoma (PEL) (Cesarman et al. 1995, 1996; Cheung, 2004b) and multicentric Castleman disease (MCD) (Soulier et al. 1995). However, as the seroprevalence of KSHV far exceeds the incidence of these diseases (even in populations known to be at higher risk), we can conclude that KSHV is necessary but not sufficient for the development of disease (Blauvelt 1999).

Complete sequencing of the KSHV genome revealed relatedness to members of the *Gammaherpesvirinae* subfamily (Neipel et al. 1997; Russo et al. 1996; Zhong et al. 1996). KSHV has been placed in the *Rhadinovirus* or gamma-2 group within this subfamily, becoming the first human herpesvirus thus categorized. The KSHV genome is ~165 kb with a central unique coding region of ~140 kb flanked on either end by variable numbers of noncoding, GC-rich repeat sequences (Lagunoff and Ganem 1997; Renne et al. 1996a).

The coding region of KSHV consists of seven blocks of conserved herpesvirus genes that align closely with those of the prototypical gamma-2 virus, herpesvirus saimiri (HVS), a virus that causes fatal T cell lymphomas in New World primates (1997 IARC Working Group; Fickenscher and Fleckenstein, 2001). These conserved genes are labeled open reading frames (ORF) 1 through 75, and most encode proteins required for production of progeny virions (transcription factors, DNA synthesis-related enzymes, as well as capsid, tegument, and envelope proteins) or proteins involved in the establishment and maintenance of latency. Interspersed between the conserved blocks are genes that are unique to gammaherpesviruses or to KSHV, labeled K1 through 15. Many of the K genes have homologs to cellular genes involved in intra- and inter-cellular signaling including cytokines, chemokines, chemokine receptors and interferon response factors (Bubman and Cesarman 2003).

Both *in vitro* and *in vivo*, KSHV is maintained as a viral episome in a primarily latent state characterized by expression of few viral gene products (Ascherl et al. 1999; Brousset et al. 2001; Davis et al. 1997; Dupin et al. 1999; Katano et al. 2000; Parravicini et al. 2000; Polstra et al. 2003; Sturzl et al. 1997). At least three viral gene products, vFLIP, vCYC, and LANA-1, are believed to comprise the minimal latency expression program of KSHV and are consistently expressed in all virally infected cells in KS, PEL, and MCD (Davis et al., 1997; Dupin et al., 1999; Sadler et al., 1999; Sturzl et al., 1997). The gene product of ORF 71, vFLIP (viral FLICE-inhibitory protein; K13) inhibits apoptosis (Djerbi et al. 1999; Thome et al. 1997) and activates NF- κ B (Liu et al., 2002). ORF 72, the viral cyclin (v-Cyclin), is a homologue of cellular D-type cyclin that can drive quiescent cells into S phase in part by inhibiting retinoblastoma protein (Rb) activity (Chang et al. 1996; Child and Mann 2001; Godden-Kent et al. 1997; Verschuren et al. 2002). In addition, v-Cyclin inactivates the cyclin-dependent kinase inhibitor p27 (Swanton et al. 1997) and promotes cytoplasmic export of human Orc1, a component of the host origin recognition complex (Laman et al. 2001). The latency-associated nuclear antigen (LANA-1; ORF 73) targets the tumor suppressor proteins p53 and Rb (Friborg et al. 1999; Radkov et al. 2000), providing both antiapoptotic and cell cycle-regulatory functions. LANA-1 is also a transcriptional modulator of various cellular and viral promoters (Renne et al. 2001) and tethers multiple copies of the viral episome to host cell chromosomes, a function indispensable for maintenance of the viral genome during cell division (Ballestas and Kaye 2001).

Two additional viral genes, K10.5 and K12, are transcribed during latency but have additional features deserving of mention. K10.5 is expressed during latency in the B cell disorders MCD and PEL, but not KS (Rivas et al. 2001). The gene product of K10.5, LANA-2, also known as viral interferon regulatory factor 3 (vIRF3), has partial homology to members of the cellular IRF family

(particularly IRF4) and two other viral IRFs, K9 (vIRF1) and K11.1 (vIRF2) (Lubyova and Pitha 2000; Rivas et al. 2001). A role for LANA-2 in inhibition of p53-induced apoptosis has been proposed, both through direct interaction with p53 (Rivas et al. 2001) and via inhibition of NF- κ B activity (Seo et al. 2004); thus LANA-2 could contribute to proliferative or neoplastic expansion of KSHV-infected B cells.

Kaposin (K12) was initially identified as an abundant latent transcript in KS tumor samples (Zhong et al. 1996); unlike other KSHV latency genes, however, K12 transcript levels increase after lytic cycle induction, suggesting a more complicated transcriptional regulation pattern. Questions awaiting elucidation include whether Kaposin utilizes different transcription units during latency and lytic replication, as well as how cell cycle and tumor type influence transcriptional regulation. The Kaposin locus encodes at least three proteins (Kaposin A, B, and C) via a translational program that is similarly complex and incompletely understood (Li et al. 2002; Muralidhar et al. 1998, 2000; Sadler et al. 1999). Transforming functions have been ascribed to the Kaposin family, particularly to Kaposin A (Muralidhar et al. 1998), and recently a role for Kaposin B in stabilization of cytokine transcripts via activation of the cellular kinase MK2 was proposed (McCormick and Ganem 2005). Overall, the latency program of KSHV enables expansion of a population of latently infected spindle cells by inducing proliferation and preventing apoptosis of infected cells as well as maintaining the viral genome in daughter cells.

In vivo, the majority of KSHV-infected spindle cells and neoplastic B cells maintain the virus as a latent infection, with only a small percentage of cells expressing lytic cycle genes (Cannon et al. 1999; Guo et al. 1997; Staskus et al. 1999, 1997; Zhong et al. 1996). The KSHV gene ORF 50, a homologue of Epstein-Barr virus (EBV) Rta, encodes a replication and transcriptional activator that is necessary and sufficient to trigger lytic replication and production of viral progeny (reviewed by West and Wood 2003). ORF 50 is one of the earliest immediate-early genes induced upon lytic reactivation in B cells and endothelial cells (EC) (Krishnan et al. 2004; Lukac et al. 1999; Sun et al. 1999) and is considered the master regulator of the switch from latency to lytic replication. In vitro, KSHV-infected cells can be induced to enter the lytic cycle by ectopic expression of ORF 50 (Lukac et al. 1999; Sun et al. 1999; Vieira and O'Hearn 2004) or by treatment with phorbol esters (Renne et al. 1996b) or sodium butyrate (Sun et al. 1996; Yu et al. 1999). The biological signals that initiate the lytic cascade are not fully understood, although hypoxia (Davis et al. 2001; Haque et al. 2003), inflammatory cytokines (IC) (Chang et al. 2000; Mercader et al. 2000; Monini et al. 1999), HIV-1 *tat* (Chang et al. 2000; Harrington et al. 1997), and CMV coinfection (Vieira et al. 2001) have all been

implicated. A role for LANA-1 in controlling lytic induction via regulation of ORF 50 expression and function has also been proposed (Lan et al. 2004).

Upon reactivation, KSHV expresses a wide repertoire of gene products in a coordinated cascade, culminating in release of progeny virions from infected cells. Chemical induction significantly increases the percentage of cells that express lytic gene products and was used by two groups for initial classification of KSHV lytic genes into kinetic classes with microarray analysis (Jenner et al. 2001; Paulose-Murphy et al. 2001). Lytic replication is essential for viral dissemination, and the observation that treatment of KSHV-seropositive AIDS patients with ganciclovir decreases the incidence of KS development suggests that lytic replication plays an important role in viral pathogenesis (Martin et al. 1999). A number of KSHV lytic genes code for regulatory proteins that could conceivably drive proliferation or transformation as well as genes that modulate apoptotic signals and recognition by the host immune system (see Moore and Chang 2003 and Direkze and Laman 2004 for recent comprehensive reviews). These genes include homologues of cellular cytokines (vIL-6/K2), chemokines (vMIP-I/K6, vMIP-II/K4, vMIP-III/K4.1), chemokine receptors (vGPCR/ORF74), interferon regulatory factors (vIRF-1/K9), antiapoptotic factors (vIAP/K7), and modulators of immune recognition (K3/MIR1 and K5/MIR2). Thus, although lytic replication is incompatible with host cell survival, reservoirs of lytic infection are thought to exert essential paracrine mechanisms that sustain tumorigenesis (see Direkze and Laman 2004 and Viejo-Borbolla et al. 2003 for recent reviews). Additionally, some speculate that a limited subset of lytic genes could be expressed under certain conditions in the absence of full lytic replication (Hayward 2003; Krishnan et al. 2004; Moses et al. 1999; Naranatt et al. 2004). Such lytic gene expression could contribute to pathogenesis without leading to host cell death resulting from production and release of progeny virus. Of particular importance here may be expression of the immediate-early gene K5, which can be expressed independent of ORF50 expression in PEL cells (Okuno et al. 2002) and experimentally infected 293A cells (Jae Jung, personal communication). K5, along with another IE gene, K3, perform a crucial immune evasion function via downregulation of MHC-1 on infected cells (Ishido et al. 2000).

3

KSHV-Infected Cell Lines Derived from Primary Effusion Lymphomas

PEL (also body cavity-based lymphomas) are KSHV-related neoplasms that can present independently of HIV infection (Cesarman et al. 1996) but are more frequent and severe in AIDS patients (Cesarman et al. 1995). Because

most PEL tumors are CD20 negative, PEL patients cannot be treated with B cell-targeted therapies; the prognosis of PEL, therefore, is poor. KSHV was first recovered by cultivation of cell lines derived from PEL (Renne et al. 1996b). Numerous clonal PEL cell lines have now been established (Arvanitakis et al. 1996; Boshoff et al. 1998; Cannon et al. 2000; Gaidano et al. 1996; Herndier et al. 1994; Katano et al. 1999; Komanduri et al. 1996; Picchio et al. 1997), about half of which are coinfecting with EBV (Boshoff et al. 1998; Carbone et al. 1996; Cesarman et al. 1996; Matsushima et al. 1999; Renne et al. 1996b; Strauchen et al. 1996). Cell lines can be readily established from PEL tumors and, unlike explanted KS spindle cells, maintain the KSHV genome in 100% of cells even after extensive tissue culture passage (Dupin et al. 1999; Fakhari and Dittmer 2002; Jenner et al. 2001; Kedes et al. 1997; Kellam et al. 1997, 1999; Paulose-Murphy et al. 2001; Rainbow et al. 1997; Rivas et al. 2001). In PEL lines, the KSHV genome is maintained as 50–150 latent episomes per cell, which is approximately 10-fold higher than in KS (Boshoff et al. 1995; Lallemand et al. 2000). Although PEL infection is predominantly latent, lytic replication can be induced by chemical induction or ectopic expression of ORF50 (Lukac et al. 1999; Renne et al. 1996a). Collectively, these characteristics have made PEL lines an extremely consistent and convenient model in which to study KSHV biology. PEL were used for the initial in vitro studies of KSHV biology, including those that yielded the viral genomic sequences, and have since been widely used to determine the kinetics of viral gene expression and mechanisms of viral latency and reactivation (Jenner et al. 2001; Lu et al. 2004; Lukac et al. 1999; Nakamura et al. 2003; Neipel et al. 1997; Nicholas et al. 1998; Paulose-Murphy et al. 2001; Russo et al. 1996; Sun et al. 1996, 1999; Zhu et al. 2004). PEL systems have also been used to develop novel KSHV-specific therapeutic strategies that may find clinical application. Klass et al. (Klass et al. 2005), demonstrated that inducing replication in PEL cells with valproate (an antiseizure medication with histone deacetylase inhibitor action) while simultaneously blocking herpesviral DNA replication with ganciclovir and phosphonoformic acid led to apoptosis in the tumor cells without increasing viral load. Godfrey et al. (Godfrey et al. 2005) tested RNA interference as a therapeutic strategy for PEL. With the use of lentivirus-delivered short hairpin RNA (shRNA) to target latency genes, inhibition of v-Cyclin and v-FLIP led to apoptosis in all four PEL lines (BC-3, BCP-1, JSC-1, and HBL-6) tested. Non-KSHV cell lines including the Burkitt lymphoma line RAMOS were unaffected by this treatment. Interestingly, LANA-1 was not as reliable a target; stable cell lines maintaining the sh-LANA vector, and consequent lower KSHV copy numbers, could be generated. Whether this result reveals a capacity for KSHV to integrate into the host genome, thus becoming LANA-independent for segregation during cell division, remains to

be established. Another interesting finding emerging from this study was the fact that LANA knockdown increased expression of ORF50, a result consistent with a recent report that LANA inhibits ORF50 expression and function (Lan et al. 2004). In a murine model, injection of the sh-v-cyclin vector prevented development of PEL-driven ascites and reduced established ascites. Other researchers have also used PEL lines to establish animal models for the study of KSHV biology and PEL tumorigenesis *in vivo* (Boshoff et al. 1998; Picchio et al. 1997; Salahuddin et al. 1988; Staudt et al. 2004; Zenger et al. 2002). In the future, such models should greatly facilitate *in vivo* screening and validation of anti-KSHV drugs that may have efficacy for KS as well as PEL.

Despite their considerable contribution to our current understanding of KSHV biology, PEL cell lines are not amenable to studying KSHV induction of transformation because the transformation event precedes cell line establishment *in vitro*. Interestingly, primary B cells and some established B cell lines can be infected with KSHV *in vitro*, but infection does not lead to prolonged maintenance of the viral genome or to cellular transformation (Bechtel et al. 2003; Blackburn et al. 2000; Naranatt et al. 2004). This is in marked contrast to B cell infection by two closely related gammaherpesviruses, EBV and RRV, both of which readily transform B cells and establish persistent infections. The reasons for this disparity are unknown, and further studies are required to clarify these issues. Additionally, PEL are of limited utility in studying the cell-specific role of KSHV in KS, where the target cell is of endothelial lineage. Therefore, development of EC-based systems that support *de novo* KSHV-infection has been essential for studying KSHV infection and gene expression patterns in non-B-lineage cells, for identifying KSHV-induced changes in EC physiology that contribute to KS pathogenesis, and for developing effective KS-targeted therapies.

4

Experimental Infection of Endothelial Cells with KSHV

Spindle cells harbor the KSHV genome *in vivo*, but all described cell lines derived from KS lesions appear to rapidly lose the KSHV genome upon serial *in vitro* passage (Aluigi et al. 1996; Ambroziak et al. 1995; Benelli et al. 1996; Boshoff et al. 1995; Herndier et al. 1994; Li et al. 1996; Staskus et al. 1997). Genome loss could be explained by an insufficiency of the episomal maintenance mechanism in endothelial-lineage cells. Others have argued that this phenomenon is artifactual, reflecting the different selective pressures inherent in tissue culture conditions versus the KS lesional microenvironment. Because explanted KS cells lose the KSHV genome after serial passage, the

development of in vitro models for KS using experimental de novo infection has proven invaluable for investigation of KSHV-EC interaction.

EC are the presumptive precursors of the characteristic spindle cells in KS lesions (Aluigi et al. 1996; Beckstead et al. 1985; Roth et al. 1988; Rutgers et al. 1986; Scully et al. 1988) although, as discussed in more detail below, whether KSHV preferentially infects lymphatic, vascular, or precursor EC is a matter of debate. Spindle cells harbor KSHV DNA in vivo (Boshoff et al. 1995; Li et al. 1996; Staskus et al. 1997), but the loss of the genome from explanted tumor cells, as well as the need to evaluate the consequences of de novo infection, stimulated efforts to develop in vitro EC-based culture systems that supported KSHV infection (Ciufo et al. 2001; Flore et al. 1998; Krug et al. 2004; Lagunoff et al. 2002; Moses et al. 1999). All EC-based KS models aim to generate age- and passage-matched infected and uninfected cultures, illuminating an important advantage over PEL cell lines that lack uninfected counterparts for use as controls. The EC-based in vitro systems described to date differ to varying degrees with respect to the protocol used and the observed consequences of infection, but key similarities have also been noted. Collectively, when evaluated in the context of the differences in experimental design, these models have yielded valuable information about KSHV biology and pathogenesis in EC. The degree to which any of these models reproduces the spindle component within the complex tumor microenvironment will be discussed further toward the end of this chapter.

Flore et al. were the first to describe infection of primary EC in vitro, using adult bone marrow-derived EC as initial targets and HUVEC as secondary recipients to demonstrate both productive infection and paracrine influences (Flore et al. 1998). Virus inoculum was prepared from concentrated TPA-induced supernatants from the BC-3 PEL line. The KSHV-exposed cultures acquired telomerase activity, exhibited extended life spans, and formed colonies in soft agar, suggesting a KSHV-mediated transformation event. However, the frequency of initial infection was low and KSHV was maintained in only a minority of cells (<10%) after serial passage. Thus, the long-term survival of these cultures was attributed to indirect influences that could also be reproduced by transferring supernatants from infected cultures to naive, uninfected HUVEC. Specifically, the type 2 VEGF receptor, KDR, was upregulated on all cells, regardless of infection status, via a KSHV-initiated paracrine effect, rendering the KDR+ cells responsive to VEGF in the culture medium. Although more recent models have allowed a higher percentage of infected cells, the paradigm whereby a subset of infected cells influences the larger microenvironment remains extremely relevant to KS pathogenesis.

Ciufo et al. describe infection of primary adult and neonatal dermal microvascular EC (DMVEC) using infectious KSHV prepared from the supernatants

of three different TPA-induced PEL lines, BCP-1, BC-3 and JSC1 (Ciufo et al. 2001). Virus was concentrated to generate high-titer inoculae allowing for a high initial infection rate. Interestingly, inoculum prepared from the JSC1 line allowed the most efficient infection, due to the production of a higher number of progeny virions and perhaps some qualitative differences between PEL lines as well. Upon infection, EC morphology changed from a cobblestone shape with a flat aspect to aggregates of cells with elongated spindle morphology. Infection of the primary DMVEC appeared to be predominantly latent; almost all converted spindle cells were LANA-1-positive, with approximately 5%–10% of cells expressing immediately early or early lytic proteins (e.g., K8, K5, ORF50, and ORF59) and 1%–2% expressing the late lytic glycoprotein K8.1A. K8.1A-positive cells were typically a subfraction of rounded up cells exhibiting CPE associated with the final stages of productive replication. In some of the K8.1A-positive or ORF59-positive cells, intranuclear inclusions resembling herpesviral replication compartments were visible. In summary, infection of primary DMVEC with JSC1-derived KSHV at high MOI led to the establishment of a predominantly latently infected cell pool that showed marked morphological changes reminiscent of KS spindle cells, while a subset of cells underwent spontaneous lytic replication with release of infectious progeny. These findings are reminiscent of KS tumor cells *in vivo*, with respect to both morphology and viral gene expression patterns. The KSHV-infected cultures could not be maintained indefinitely, however, both because of a chronic loss of cells from lytic infection and failure of the latently infected cells to be maintained for more than a few cycles as a population of dividing cells that maintained the viral episome. Addition of uninfected cells to the cultures at a 1-to-10 ratio provided fresh targets for infection, providing a means to generate additional infected cultures without establishing new PEL-initiated infections. Thus, multiple rounds of *de novo* infection, as opposed to long-term maintenance of latently infected cells, define this culture model.

Similar protocols have since been used to generate KSHV-infected EC cultures to examine patterns of viral (Krishnan et al. 2004) or host (Hong et al. 2004; Naranatt et al. 2004; Poole et al. 2002; Wang et al. 2004) gene expression by microarray analysis, RT-PCR, and/or immunostaining after *de novo* infection with PEL-derived virus. Krishnan and colleagues (Krishnan et al. 2004) were the first to comprehensively examine viral gene expression patterns in primary EC after *de novo* infection. In this report, adult DMVEC were infected with concentrated BCBL-1-derived KSHV and the kinetics of latent and lytic gene expression were examined by whole genome array (Celonex HHV8 viruChip), RT-PCR, and immunostaining at time points from 30 min to 5 days postinfection (PI). This report revealed a number of interesting findings. Expression of a limited set of immediate-early (IE) and early (E) lytic genes was

initiated concurrently with latent genes in the majority of cells immediately after infection, with a sharp decline thereafter. Compared to TPA-induced BCBL cells, only a limited number of ORF50-activated genes were expressed, and the majority of genes involved in DNA replication and viral assembly were not expressed. Of particular interest, the IE gene K5/MIR2 was expressed at early time points and continued to be well expressed after a decline in other IE and E genes. Another interesting finding was confirmation of the absence of LANA 2 (ORFK10.5) expression in primary EC. Expression of a limited set of IE and E genes with immune evasion and antiapoptotic function, with a subsequent decline thereafter, may play a crucial role in vivo in allowing establishment of infection and tumor initiation/progression. Signals that determine which cells progress through a full lytic cycle and produce infectious progeny, versus those that establish latency, remain to be determined; indeed their elucidation may require the multifactorial tumor environment to be more fully represented in in vitro KS models.

Host EC gene expression has been evaluated after only 2 and 4 h PI (Naranatt et al. 2004), after several days (Hong et al. 2004; Wang et al. 2004), or after 3 weeks (Poole et al. 2002). Poole et al. (Poole et al. 2002) also performed microarray analysis after reseeding of infected cultures with uninfected cells (Poole et al. 2002). Naranatt and colleagues (Naranatt et al. 2004) examined gene expression in KSHV-infected primary DMVEC with Affymetrix HG-U133A gene arrays as early as 2 and 4 h PI and discovered a significant early reprogramming of the host transcriptome that included alterations in genes involved in signaling, apoptosis, transcription, host defense, cell cycle, metabolism, inflammation, angiogenesis, and tumorigenesis. The authors also evaluated infected fibroblasts and the B cell line BJAB, to create a database that illuminated both cell type specific and common responses to infection. The study by Poole and colleagues (Poole et al. 2002) differed in that the authors' aim was to interrogate host reprogramming after establishment of a latent infection and after exposure of latently infected cells to TPA; hence, cells were harvested 3 weeks PI as well as 2 weeks after reseeding infected cells with new uninfected targets. Clontech Human Atlas or Incyte Human UniGemV2.0 cDNA arrays were used, with RT-PCR analysis confirming changes in selected individual genes. This study revealed that, even after establishment of latency, KSHV-infected cells exhibited a profound alteration in gene expression patterns; between 1.4% and 2.5% of genes represented were significantly upregulated or downregulated. Of particular interest was the induction of interferon-induced genes, genes involved in cell signaling and angiogenesis, and genes involved in cell cycle progression and apoptosis. The studies by Hong and colleagues and Wang and colleagues (Hong et al. 2004; Wang et al. 2004) were specifically designed to evaluate lymphatic reprogram-

ming and are discussed below, along with a similar study using immortalized EC (Carroll et al. 2004).

Primary infection of EC has also been used to study KSHV binding and entry and the signaling pathways induced in the earliest phases of infection (Naranatt et al. 2003). This study identified a role for integrin $\alpha_3\beta_1$ -FAK-dependent phosphatidylinositol 3- (PI 3-) kinase activation in KSHV entry and a role for the PKC- ζ -MEK-ERK signaling cascade during the earliest stages of KSHV infection. Activation of a mitogenic cascade during KSHV entry may have important implications for establishment of latency and the division of latently infected cells, as well as creation of a host microenvironment conducive to the expression of immediate early lytic cycle proteins.

A recent report by Krug et al. is worthy of mention because infection was performed at low MOI to specifically explore the ability of latently infected EC to replicate as well as the ability of infected EC to produce infectious virus for dissemination via de novo infection (Krug et al. 2004). Pooled neonatal DMVEC were infected with KSHV derived from TPA-induced BCBL-1 cells. Although less than 5% of cells were initially infected, infection spread primarily because of proliferation of latently infected cell with episome maintenance. A limited contribution by de novo infection from virions produced from the small percentage that entered the lytic cycle was also noted. Spread of infection could not be blocked by treating cultures with compounds that block herpesviral lytic replication; therefore, proliferation of latently infected cells characterizes this culture system. A growth advantage for LANA-1-positive cells as compared to uninfected cells was noted, and cell spindling with loss of contact inhibition after postconfluent growth was observed. Thus, of all primary infection protocols, this is the most similar to the system described by Moses et al. that is discussed in Sect. 5. Of note, infection of the primary EC induced expression of cellular antiviral genes, specifically dsRNA-activated protein kinase (PKR) and 2',5'-oligoadenylate synthetase (2'5'-OAS), a phenotype that could be reproduced by IFN- γ treatment. IFN- γ treatment was also effective in preventing lytic activation and viral replication. However, because infection was initiated at low MOI and was maintained primarily by proliferation of latently infected cells, induction of antiviral genes did not eliminate viral infection in this system. Other protocols for infection of primary cells that utilize high-titer inoculae may induce higher levels of these host-defense genes, which may in turn interfere with establishment and maintenance of infection.

The life span of primary EC *in vitro* is limited because of replicative senescence. To overcome this limitation, some investigators have employed life-extended EC prepared by ectopic expression of human telomerase or genes from other transforming viruses (Lagunoff et al. 2002; Moses et al. 1999). Expression of these genes does not induce laboratory evidence of transformation

other than extending the length of time cells can be serially propagated in culture. A system described by Moses et al. was the first to use life-extended EC for KSHV infection and the first to describe cultures in which the majority of cells became infected and maintained the genome in a predominantly latent state (Moses et al. 1999). In this model, adult primary DMVEC are immortalized by retroviral transduction of the E6 and E7 genes of human papillomavirus (HPV) type 16. The life-extended cells exhibit no overt signs of transformation (i.e., they become contact inhibited at confluence and do not form colonies in soft agar) but with serial passage can be maintained significantly longer than their primary counterparts. HPV E6 and E7 are known to have significant effects on the cell cycle, most notably through targeting p53 and Rb. E6 participates in the ubiquitination and degradation of p53 (Werness et al. 1990), and E7 binds and sequesters Rb (Howley et al. 1989) and induces Rb phosphorylation (i.e., inactivation) (Zerfass-Thome et al. 1996). These are two pathways that KSHV latent genes also inhibit; LANA-1 disrupts the function of both p53 (Friborg et al. 1999) and Rb (Radkov et al., 2000), and v-Cyclin induces Rb phosphorylation via activation of CDK-6 (Godden-Kent et al. 1997). In addition, LANA and p53 colocalization is observed in KSHV-associated tumors *in vivo* where p53 is usually wild type, suggesting that LANA-mediated p53 inactivation is important for tumorigenesis (Katano et al. 2001). Therefore, immortalization of DMVEC by E6/E7 expression may augment some of the alterations in cellular physiology that are themselves induced by KSHV, thus creating a cellular microenvironment conducive to KSHV infection. It cannot be ruled out, however, that these HPV gene products complicate results obtained when using this culture system for studies of KSHV biology. Thus, key findings obtained with E6/E7-immortalized DMVEC have been verified in primary cells and KS tissue (McAllister et al. 2004; Moses et al. 1999, 2002a; Raggo et al. 2005).

For studies performed with the E6/E7-DMVEC model to date, infectious KSHV has been derived from unconcentrated TPA-induced BCBL-1 cultures, yielding a relatively low-titer inoculum and thus the expectation of a low percentage of initially infected cells. Indeed, evaluation of LANA-1 expression by IFA at early times (12 h) PI revealed that not more than 10% of EC in a treated culture were KSHV infected (Moses et al. 1999). LANA expression increased with time, such that by 14 days PI up to 80% of cells in a KSHV-exposed culture were infected. PCR for the KSHV genome at days 7 and 14 PI revealed increased intensity of the amplified product by day 14, supporting evidence of virus spread. A similar trend was seen with expression of the early lytic protein ORF 59: At 1 week PI <1% of cells were ORF 59 positive, but by 8 weeks PI up to 5% of cells expressed this lytic marker. Expression of the late lytic glycoprotein K8.1A/B followed similar expression kinetics, though

to a consistently 5- to 10-fold lesser degree than ORF 59. Lytic replication could be induced in infected cells by treatment with TPA, sodium butyrate, or corticosteroids, but never in more than 40% of LANA-positive cells.

Before exposure to KSHV, E6/E7-immortalized DMVEC retained a classic cobblestone appearance. KSHV-infection, however, induced marked changes in cellular morphology reminiscent of the spindle cells observed in KS lesions including elongated cells with oval cell bodies, uniformly narrow elongated cells, and extremely narrow light-refractile cells displaying scattering. A low percentage of rounded up cells that tended to detach from the monolayer were also observed; the nuclei of these cells as well as the nuclei of extremely spindled cells displayed intranuclear inclusions resembling typical herpesviral CPE. The extent of morphologic change within virus-exposed cultures increased with time PI and correlated strongly with the percentage of KSHV-infected cells, suggesting a direct effect of the virus on cell morphology. Evaluation of gene expression in concert with morphology revealed the following: LANA-1 expression was sufficient for spindling; ORF59-positive cells were spindle-shaped, with a proportion displaying intranuclear inclusions, and all rounded cells were strongly ORF59 positive; rare K8.1A/B positive cells displayed severe spindling but were more frequently rounded. The presence of infectious virions in conditioned supernatants that could transfer infection to naive cultures, albeit at a low MOL, confirmed that DMVEC could support a fully permissive replication cycle. The relative ratios of ORF73- to ORF59- to K8.1A/B-positive cells at any one time suggested that the majority of cells harbored latent infection and that completion of the lytic cycle occurred in only a fraction of the lytic cell population. These observations are very similar to those reported by Ciuffo and colleagues on infection of primary DMVEC (Ciuffo et al. 2001).

Evaluation of KSHV gene expression and cell morphology suggests that the attainment of completely infected cultures in the E6/E7-DMVEC model reflects a combination of the proliferation of latently infected cells and de novo infection by EC-generated virus. Once all cells are infected and naive viral targets are absent, latently infected cells continue to proliferate with only a minor fraction of cells lost due to productive lytic infection. Infected cultures can be expanded by passaging, but the genome is best maintained when low split ratios are used; presumably a delicate balance exists between cell division and episome replication and segregation to daughter cells. A key difference between the immortalized DMVEC and primary EC is the increased length of time for which latently infected E6/E7-DMVEC can be passaged with genome maintenance. It is possible that the presence of E6/E7 allows for a degree of episome maintenance more akin to what is seen in PEL cells that, unlike KS tumor cells, are able to maintain the KSHV episome *in vitro* as well as *in vivo*.

The latent gene ORFK10.5/LANA-2 that inhibits p53 function is well expressed in PEL cell but not in primary DMVEC (Krishnan et al. 2004; Parravicini et al. 2000); possessing multiple ways to disable this tumor suppressor protein may be a reason for efficient episome maintenance in PEL cells. Distinct from their role as oncoproteins, E6 and E7 are also known to be required for stable maintenance of HPV episomes in undifferentiated human keratinocytes (Thomas et al. 1999). The HPV oncogenes in the immortalized DMVEC may assist in episome maintenance by duplicating the function of LANA-2 or by acting as surrogates for other currently unappreciated mechanisms occurring in the lesional microenvironment.

Interestingly, after several weeks in culture, there is a decrease in both spontaneous ORF 59-expression and inducibility of lytic cycle proteins by chemical induction in KSHV-infected E6/E7-DMVEC. Thus, a predominantly latent infection is established, possibly by selection of clones that are resistant to lytic induction but that can propagate as continually dividing, latently infected cells. In such cultures, CD31 is strongly expressed on the LANA-positive cells, suggesting that K5 is not expressed and thus a true latency exists (Mandana Mansouri and Klaus Früh, unpublished observations).

As described above, the E6/E7-DMVEC model appears to represent two distinct stages: one in which the viral infection and genome spread occurs via both lytic and latent means and a second where latently infected cells survive for multiple passages and maintain the genome in a latent state. When such cells are not passaged before achieving tissue culture confluence, a third state is generated, characterized by the continued postconfluent growth of the cells into three-dimensional foci. Importantly, uninfected cells grow to confluence and enter a quiescent state under similar conditions. Postconfluent growth reflects loss of contact inhibition, one of the hallmarks of cellular transformation. Infected cells are also able to form colonies in soft agar, a measure of anchorage-independent growth, and form tumors in mice when injected into the tail base in a matrigel solution (Shane McAllister and Ashlee Moses, unpublished observations). Krug and colleagues (Krug et al. 2004) similarly report that when primary DMVEC are infected at low MOI with subsequent spread of infection primarily through division of latently infected cells, loss of contact inhibition is observed. The piled-up aggregates of infected cells reported by Ciufu and colleagues (Ciufu et al. 2001) are also reminiscent of three-dimensional focus formation. Thus acquisition of a transformed phenotype is not unique to the E6/E7 DMVEC system, although the potential for additional contributions from the HPV proteins should be considered.

Latently infected E6/E7-DMVEC have been used in gene expression profiling studies to examine KSHV-induced cellular gene reprogramming and identify potential therapeutic targets for KS. These studies have employed

cDNA arrays (Moses et al. 2002b) as well as Affymetrix U95A and U133A and B GeneChips (Moses et al. 2002a; Raggio et al. 2005) and have interrogated several different KSHV-infected cultures relative to age- and passage-matched uninfected controls. For all of these studies, KSHV-infected DMVEC were infected with BCBL-1-derived KSHV at low MOI and passaged when confluent at low split ratios. Cells were harvested for microarray analysis when immunofluorescent staining of parallel cultures revealed that >90% of KSHV-infected cultures were LANA positive. This typically took 3–4 weeks and encompassed approximately five to seven tissue culture passages. A minimum of two biological replicates were used for each comparison. The complete data sets from these different microarray experiments are available online (ohsu.edu/vgti/fruh.htm), and further details can be found in the specific papers referenced. To date, this database has been used to identify a handful of potential targets for KS chemotherapy (McAllister et al. 2004; Moses et al. 2002b; Raggio et al. 2005). Briefly, the receptor tyrosine kinase c-Kit was identified as a gene induced by KSHV with cDNA arrays (Moses et al. 2002b) and confirmed with Affymetrix arrays, RT-PCR analysis, and immunofluorescence, the latter being on primary DMVEC. An independent analysis of gene expression in primary DMVEC with U133A GeneChips also reported KSHV induction of c-Kit (Hong et al. 2004). A role for c-Kit in proliferation and post-confluent growth of KSHV-infected DMVEC was then demonstrated with both a pharmacological inhibitor of c-Kit [Imatinib Mesylate, Gleevec; formerly STI 571 (Moses et al. 2002b)] and gene knockdown approaches (Moses et al. 2002a). These data, combined with evidence of c-Kit expression in KS tumors (Pantanowitz et al. 2004), contributed to a recent clinical trial that demonstrated the efficacy of Imatinib Mesylate as a therapeutic regimen for KS (Koon et al. 2004). Because both c-Kit and PDGF-R are expressed in KS, the relative importance of these two targets remains unclear. However, the study is notable because it was the first to attempt to identify novel drug targets for KSHV with microarrays and to test the efficacy of a pharmacologic agent in a disease model (Jenner and Young 2005). The same conceptual approach was used to identify two KSHV-induced cellular proteins, RDC-1 and Neurtin, with novel oncogenic properties (Raggio et al. 2005). RDC-1 was also identified as one of the most highly induced genes in the microarray studies performed on primary DMVEC by Poole and colleagues (Poole et al. 2002). E6/E7-DMVEC were recently used in a proteomics-based screen to identify KSHV induction of the enzyme heme oxygenase-1 (HO-1) (McAllister et al. 2004). HO-1 expression in KS tissue was confirmed by the authors and by a recent SAGE study (Cornelissen et al. 2003). Inhibition of HO-1-induced proliferation of infected cells by treatment with mesoporphyrin compounds may, on further study, offer an additional treatment option for KS (McAllister et al. 2004; Yarchoan 2004).

A second efficient culture system for studying KSHV biology based on the use of immortalized EC was described by Lagunoff and colleagues (Lagunoff et al. 2002). In this model, human neonatal DMVEC were immortalized by retroviral transduction of the telomerase reverse transcriptase subunit (hTERT) (Venetsanakos et al. 2002). The telomerase-immortalized microvascular endothelial cells (TIME cells) retained a normal karyotype as well as many of the properties of the primary cells from which they were derived, including expression of CD31 and $\alpha_v\beta_3$ -integrin, LDL uptake, and tubule formation in matrigel. TIME cells grow well when serially passaged and become contact inhibited at confluence, with the only morphologic change being a mild cell spindling.

TIME cells are infected at a high MOI with KSHV concentrated from TPA-induced BCBL-1 supernatants. Nearly all TIME cells are LANA positive at 48 h after infection, and latency is the predominant outcome; only about 1% of infected cells express the early lytic protein ORF 59, and a smaller subset express the late structural glycoprotein K8.1. This system thus accurately reflects the state of the viral genome in vivo (Staskus et al. 1997) and is reminiscent of what has been observed after de novo infection of primary and E6/E7-DMVEC (Ciufo et al. 2001; Moses et al. 1999). In addition, because of the high frequency of initial infection, TIME cells provide a valuable system for studying early events in de novo infection. With increasing time PI, however, there is a rapid reduction in the infected TIME cell population, such that by tissue culture passage 7, less than 0.1% of cells are infected. Such loss of latently infected cells suggests inefficient maintenance of the viral episome and resembles what is seen with explanted KS spindle cells. However, because a percentage of TIME cells are lytically infected, infectious virus can be serially transferred or cultures can be maintained by addition of uninfected cells. An advantage of the TIME system is the ease with which TIME cells can be cultivated and infected with high efficiency. In addition, potential effects from oncoproteins such as E6 and E7 are not a concern in this model.

While TIME cells establish a predominantly latent infection, they can be induced to lytically reactivate by infection with an ORF50-expressing adenovirus vector (Bechtel et al. 2003). Using this system, Glausinger and Ganem (Glausinger and Ganem 2004) demonstrated that lytic KSHV infection strongly inhibits host gene expression by accelerating global mRNA turnover. Shut-off is mediated by the viral SOX (shutoff exonuclease) protein, the product of the HSV alkaline exonuclease homologue ORF37. The TIME cells were subsequently used to demonstrate that a subset of host transcripts, including IL-6 and the IL-1 type 1 receptor, escape host shut-off (Glausinger and Ganem 2004).

TIME cells express very little or no Prox-1 protein before KSHV infection, suggesting that the original immortalized clone was derived from blood vascular, as opposed to lymphatic, endothelium. Prox-1 is required for expression of two key markers that differentiate lymphatic from vascular endothelium, VEGFR3 and podoplanin (Wigle and Oliver 1999). TIME cells have thus also proved useful for investigating the hypothesis that KSHV infection drives EC to a more lymphatic phenotype, a hypothesis supported by the robust expression of lymphatic EC markers on KS spindle cells (Jussila et al. 1998; Skobe et al. 1999). Carroll and colleagues (Carroll et al. 2004) used RT-PCR and cDNA microarray techniques to investigate expression of genes specific to lymphatic EC after KSHV infection of TIME cells and reported significant induction of such genes including Prox-1, VEGFR3, podoplanin, and LYVE-1. The microarray studies were performed after 24, 48, and 96 h of infection; 147 genes (about 1% of genes on the array) were significantly induced (>1.8 fold; $P < 0.001$) at all time points and 61 genes significantly repressed. This list comprises another valuable data set with which to examine KSHV reprogramming of the host transcriptome, particularly at early times PI and when considering the blood vascular phenotype of the uninfected controls. Array analysis performed with primary cells may reflect a mixed population of both blood and lymphatic EC, because both types can be present in primary cultures (Makinen et al. 2001). In addition to the differential expression of lymphatic markers observed in KSHV-infected TIME cells, other interesting findings included significant upregulation of host IL-6 and significant downregulation of IL-8. Induction of IL-6 expression is consistent with the finding that IL-6 escapes host shut-off; loss of IL-8 protein expression in KSHV-infected E6/E7-DMVEC stimulated with IL-1 has also been observed (Ashlee Moses and Michael Jarvis, unpublished observations).

Telomerase-immortalized KSHV-infected DMVEC have also been described by Tomescu and colleagues (Tomescu et al. 2003). The cells used in this study, TIME-T4 cells, were derived from a different parental cell than that of Lagunoff et al. but were transduced with the same retroviral hTERT expression vector. TIME-T4 cells were infected with concentrated BCBL-1-derived virus or via coculture with BCBL cells. The TIME-T4 cells respond differently to KSHV infection than the original TIME cells in that a dramatic spindling was observed as soon as 24 h PI. This may be due to an inherent difference in the immortalized cell clone or to quantitative or qualitative differences in the inoculae used. The T4 cells demonstrated downregulation of major histocompatibility complex (MHC) class I and the adhesion molecules ICAM-1 and CD31, within 48 h of infection, indicating that this system is extremely useful for the study of KSHV immune evasion mechanisms in EC. The effective downregulation of MHC1 suggests that the

T4 cells were expressing latent and IE lytic genes concurrently, as suggested by the studies of Krishnan and colleagues in primary cells (Krishnan et al. 2004). TIME T4 cells should prove valuable for determining the kinetics of IE expression in EC, particularly for K3 and K5, as well as the window of time after their downregulation for which MHC1 and adhesion molecule surface expression remains functionally compromised.

The above text contains multiple references to gene expression profiling experiments performed on KSHV-infected EC. Taking into account the differences in experimental design, including cells and virus origin, infection and culture conditions and the microarray platforms used, it is not surprising that the correlation between these data sets is modest. Genes that are commonly induced could reveal patterns that are general responses to virus infections, as well as genes so tightly linked to KSHV pathogenesis that their dysregulation transcends differences in experimental design. On the other hand, differences may reveal valid temporal changes in gene expression or culture-specific events. Analyses performed at early times PI would no doubt include host genes deregulated by those early lytic viral genes that may be transiently expressed, whereas those performed after many tissue culture passages (with episome maintenance) would more reflect the influences of latent gene expression, as well as the delayed effects of those early events PI. Collectively, these studies should be viewed as a valuable database from which to further analyze links between KSHV pathogenesis and cellular gene expression and function. Valuable progress has already been made in this regard.

5 Parental Lineage of Spindle Cells

Identification of the cellular origin of KS lesional spindle cells has been a matter of ongoing debate. Spindle cells express EC, smooth muscle cell, macrophage, fibroblast, and dendritic cell markers (Kaaya et al. 1995; Regezi et al. 1993b) but are generally accepted to originate from an EC precursor. In addition to expressing markers of blood vascular endothelium, KS spindle cells express several markers specific for lymphatic endothelium, including VEGF-R3 and podoplanin (Jussila et al. 1998; Skobe et al. 1999; Weninger et al. 1999). These observations suggest either that KSHV preferentially infects lymphatic endothelium *in vivo* or that KSHV infects precursor EC, lymphatic EC (LEC), or blood vascular EC (BEC) and drives the gene expression profile to a more convergent one, where lymphatic-lineage markers are induced or retained. As discussed in detail in this chapter, KSHV infects BEC *in vitro*; if, however, LEC were the preferred target *in vivo*, this might in part explain

the inability of KSHV-infected EC to maintain the genome in tissue culture for extended periods. Wang et al. recently infected both LEC and BEC with KSHV and found with quantitative PCR that KSHV genomes were maintained at higher copy number in LEC (Wang et al. 2004). This same group compared gene expression profiles of nodular KS samples and normal skin with Affymetrix U133A arrays and developed a KS expression signature of 1,482 genes by removing genes expressed at similar levels in both KS and normal dermis or epidermis. Using expression profiles similarly generated from purified LEC and BEC, the authors found that although both LEC and BEC markers were present in KS tissue, the KS expression signature was more like that of the LEC. Interestingly, infection of LEC and BEC led to a convergence of their profiles such that they were more like each other than the uninfected counterparts. Hong and colleagues (Hong et al. 2004) compared gene profiles of primary DMVEC at day 7 PI and found significant upregulation of key lymphatic lineage-specific genes after KSHV infection including Prox1, LYVE-1, reelin, follistatin, desmoplakin, and leptin receptor. In addition, retroviral transduction of BEC with LANA led to induction of Prox1, the master gene responsible for lymphatic vessel development (Wigle and Oliver 1999). This study is in agreement with a study done in TIME cells, an immortalized BEC line, reporting KSHV induction of lymphatic-lineage markers (Carroll et al. 2004).

Collectively, these studies suggest that *in vitro* KSHV induces a transcriptional drift in BEC and LEC toward a more convergent phenotype. Because commercial EC preparations contain both LEC and BEC, the relative ratios in cell preparations used in different laboratories may have some bearing on the observed outcome of infection. Regarding immortalized EC, the BEC lineage of TIME cells was recently established (Carroll et al. 2004). On the other hand, E6/E7-immortalized EC clones that are KSHV permissive appear to have a more LEC-like phenotype; genes for LYVE-1, podoplanin, VEGFR-3, leptin receptor, oncostatin M receptor, c-MAF, and reelin are all expressed (Patrick Rose and Ashlee Moses; unpublished observations). Because KSHV genomes appear to be better maintained in LEC than BEC (Wang et al. 2004), these findings may contribute to the ability of E6/E7-DMVEC to maintain the KSHV episome for long periods of time. The relevance of the above studies to KS tumors is clearly the appreciation that lymphangiogenic molecules are involved in KS pathogenesis; if KSHV infection of EC results in a cell type with characteristics of both vascular and lymphatic EC, understanding this unique tumor phenotype will be important for understanding the disease and developing effective clinical approaches for KS therapy.

6 A Model of KS Lesion Progression

The observed frequency of immunoreactivity for KSHV markers in EC infected in vitro with a limited panel of antibodies is LANA \gg ORF 59 \gg K8.1 (i.e., latent \gg early lytic \gg late lytic). As described elsewhere in this chapter, these ratios have been observed in studies using both primary and immortalized EC and different viral infection protocols. These ratios also accurately reflect gene expression patterns found in KS lesions by immunohistochemistry and in situ hybridization (Parravicini et al. 2000). Two theoretical outcomes of lytic reactivation could account for the relative excess of early lytic versus late lytic gene expression. First, if all cells activated to the lytic cycle consistently support viral DNA replication with assembly and release of infectious progeny, then all early lytic-positive cells would, in time, progress to cell lysis and death. Alternately, if only a fraction of lytically activated cells produce infectious progeny, then a pool of cells must exist that express several IE and early genes without supporting productive viral replication. Evidence that antiherpes agents such as ganciclovir and foscarnet, which inhibit lytic but not latent herpesviral infection, can improve the clinical outcome of KS strengthens the assertion that ongoing lytic gene expression is involved in KS pathogenesis (Glesby et al. 1996; Martin et al. 1999; Mocroft et al. 1996). If every lytic event leads to cell death, then a continual pool of new targets for de novo infection must be recruited; on the other hand, limited expression of IE and E genes would allow for immune evasion and antiapoptotic mechanisms, as well for as the angiogenic and chemoattractant properties of lytic genes, to contribute to tumor formation without a net loss of cells. The clinical responsiveness of KS to drugs that block proliferation of latently infected cells (Koon et al. 2004) indicates that lytic reactivation is not the only mechanism driving KS lesion progression. Accumulation of latently infected cells in KS lesions is due to proliferation and tissue invasion of latently infected cells. The rapid loss of the KSHV genome on culture of explanted spindle cells may reflect an insufficiency of the episomal maintenance machinery in endothelial lineage cells. It is unknown whether this occurs in vivo when virally infected spindle cells are stimulated to divide. Loss of the cytoprotective functions of some KSHV genes could lead to apoptotic cell death of spindle cells after cell division without episomal maintenance. Another possibility is that spindle cells that have lost the viral genome could become targets for reinfection; multiple rounds of reinfection could contribute to the accumulation of mutations leading eventually to the outgrowth of truly transformed clonal populations of spindle cells.

The lack of a well-characterized animal model currently precludes the in vivo examination of KSHV deletion mutants, so the specific gene expression

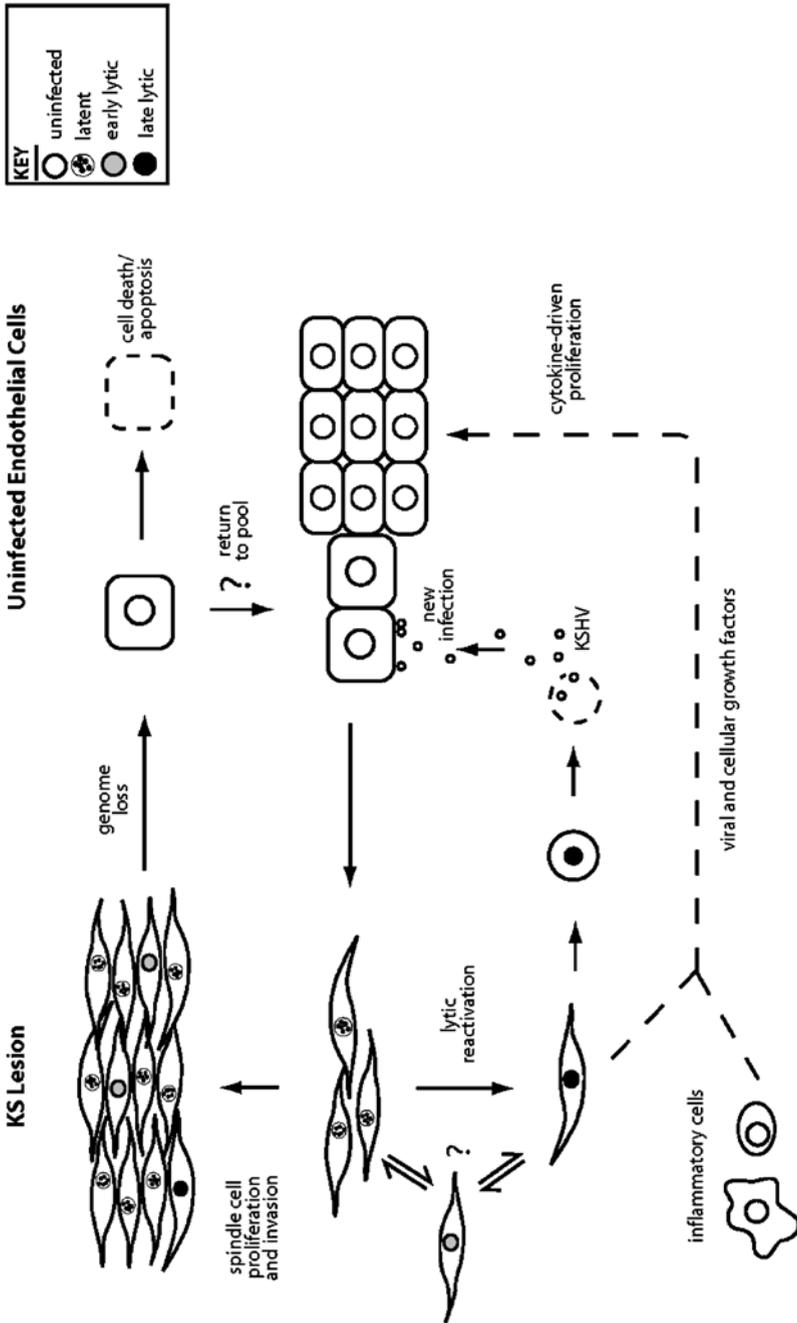


Fig. 1 The model of KS lesion progression presented here is based on recent clinical advances in KS chemotherapy and draws together multiple theories of KS that by themselves cannot account for the complexities of KS lesions: The accumulation of spindle cells as KS lesions progress is driven by both continuous de novo infection and proliferation of latently infected cells. Lytic reactivation results in both the production of infectious virus but also paracrine signaling that contributes to lesion progression by inducing proliferation of uninfected neighboring cells. Loss of the KSHV genome on multiple rounds of cell division may be followed by repeated rounds of infection, contributing ultimately to the outgrowth of truly transformed clones that have accumulated multiple mutations. It is currently unknown whether proliferating spindle cells lose the KSHV genome in vivo

requirements for KS disease states must be inferred indirectly. The clinical responsiveness of KS to drugs that block herpesvirus lytic cycle, and to drugs that block proliferation of latently infected cells, indicates that both of these mechanisms can drive KS lesion establishment and progression, perhaps cooperatively (Fig. 1).

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KSHV After an Organ Transplant: Should We Screen?

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1	Introduction	246
2	Mechanisms of Posttransplant KS Development in Solid Organ Transplant Recipients	247
2.1	Incidence of Posttransplant KS in Solid Organ Transplant Recipients	247
2.2	KSHV Reactivation After Organ Transplantation	248
2.2.1	Kidney Transplantation	248
2.2.2	Other Types of Organ Transplantation	249
2.3	KSHV Contamination After Organ Transplantation	250
2.3.1	Kidney Transplantation	250
2.3.2	Other Types of Organ Transplantation	251
2.3.3	KSHV Serology in the Context of Seroconversion	252
3	Risk Factors Associated with Posttransplant KS Development in Solid Organ Transplant Recipients	253
4	KSHV Screening and Monitoring in the Context of Solid Organ Transplant Recipients	254
4.1	KSHV Screening	254
4.2	KSHV Monitoring	254
4.3	Limitations	256
	References	257

Abstract The incidence of Kaposi sarcoma (KS) related to Kaposi sarcoma-associated herpesvirus (KSHV/HHV-8) after organ transplantation is 500–1,000 times greater than in the general population, and its occurrence is associated with immunosuppressive therapy. The reported incidence of posttransplant KS ranges from 0.5% to 5%, depending on the patient's country of origin and the type of organ received, mainly after renal transplantation. Posttransplant KS is caused by two possible mechanisms: KSHV reactivation in patients who were infected before the graft and KSHV contamination from the infected organ's donor to the recipient. KSHV reactivation appears to play a greater role in the risk of KS than incident infections. However, some studies, with findings based not only on serological data but also on molecular tracing of

the viral infection, have shown that organ-related transmission of KSHV could be more common than previously thought and associated in some cases with severe KSHV-related disease. Precise estimates of KSHV seroprevalence in the organ donor and recipient populations in different countries are lacking. However, studies have reported seroprevalences among donors and recipients that are similar to those among the general population of the country considered. Many studies have suggested the potential utility of screening of KSHV antibodies among organ donors and recipients. However, to date the results of these studies have argued in favor of KSHV screening, even in low-KSHV infection prevalence countries, not to exclude the graft but to have the KSHV status information in order to have the opportunity to monitor, clinically and biologically, patients at risk for KSHV-related disease development. The detection of KSHV antibodies could be done in the days after the transplantation and the results transmitted to the physicians retrospectively. In conclusion, the question of screening donors and recipients for KSHV, even in low-KSHV infection prevalence countries, is still debated, and prospective studies are needed to evaluate the benefit of pre- and posttransplantation strategies.

1 Introduction

Despite great advances in stem cell and organ transplantation, opportunistic infections are a major cause of morbidity and mortality for transplant recipients. Herpesviruses remain latent in the body after primary infection and can reactivate under immunosuppression conditions, such as after transplantation, making them particularly important pathogens in transplant recipients. As no effective antiviral drug is yet available for EBV and KSHV/HHV-8, recovery of the host immune response against these viruses is crucial in controlling virus-associated tumors in transplant recipients. Skin cancers are the most common malignant conditions in transplant recipients (Euvrard et al. 2003). Focusing on KSHV, the incidence of Kaposi sarcoma (KS) after organ transplantation is 500–1,000 times greater than in the general population (Harwood et al. 1979; Woodle et al. 2001). The occurrence of KS among transplant recipients is associated with immunosuppressive therapy, as evidenced by the remission of KS lesions after the reduction or withdrawal of immunosuppressive therapy (Nagy et al. 2000). The reported incidence of posttransplant KS ranges from 0.5% to 5%, depending on the patient's country of origin and the type of organ received (Jenkins et al. 2002; Qunibi et al. 1988) (Table 1).

Posttransplant KS develops in solid organ transplant recipients, mainly after renal transplantation, and is caused by two possible mechanisms: KSHV reactivation in patients who were infected before the graft and KSHV contamination from the infected organ's donor to the recipient. The proportion

Table 1 Incidence of KS in series of kidney transplant recipients from different countries

Series origin	Incidence of KS (%)
Germany (Behrend et al. 1997)	0.26
Spain (Gomez dos Santos et al. 1997; Munoz et al. 2002)	0.2–0.5
USA (Penn 1997)	0.5
Canada (Shepherd et al. 1997)	0.6
Italy (Montagnino et al. 1996)	0.7–3.3
France (Farge 1993; Frances 1998)	0.4–2.4
Israel (Shmueli et al. 1989)	2.4
Turkey (Ecder et al. 1998)	3
South Africa (Margolius et al. 1994)	4–5
Saudi Arabia (Qunibi et al. 1988)	4–5.3

KS, Kaposi sarcoma

of cases caused by each of these mechanisms varies significantly in relation to the prevalence of KSHV antibodies in the populations studied. In populations with a high prevalence of infection, such as in Italy, posttransplant KS was mainly caused by reactivation of KSHV, whereas in areas of lower prevalence, such as Switzerland, it was due to primary infections (Parravicini et al. 1997; Regamey et al. 1998). However, some studies, with findings based not only on serological data but also on molecular tracing of the viral infection, have shown that organ-related transmission of KSHV could be more common than previously thought. Moreover, other routes of transmission, such as blood products, could not be excluded as a possible source of contamination.

2 Mechanisms of Posttransplant KS Development in Solid Organ Transplant Recipients

2.1 Incidence of Posttransplant KS in Solid Organ Transplant Recipients

The incidence of KS in transplant patients depends mainly on the epidemiology of KSHV in the population involved and on the type of transplantation.

There is a 1%–3% risk of KS with kidney transplantation (Table 1), the median interval to diagnosis of KS being 29–31 months (Frances et al. 1999; Rabkin et al. 1999). In Saudi Arabia, where there is a higher prevalence of

KSHV, the transplantation risk of KS rises to 3%–5% (Qunibi et al. 1988). It may also reach high figures in transplant patients reported from other ethnic groups with a high incidence of KSHV infection, such as Mediterranean and Middle Eastern patients. For example, in one series, the overall incidence among transplant patients was 0.78% but up to 4% among those of Jewish or Mediterranean origin (Harwood et al. 1979). Another key factor is the type of transplantation. Incidence ranges from 0.2% to 2.8% in liver recipients (Besnard et al. 1996; Colina et al. 1996) and from 0.41% to 11% in heart transplant patients (Briz et al. 1998; Frances et al. 1991; Goldstein et al. 1995; Lanza et al. 1983; Zahger et al. 1993); it was 2% after kidney-pancreas transplantation (Kaufman et al. 1999), and just two case reports on lung recipients have been published (Sachsenberg-Studer et al. 1999; Sleiman et al. 1997). In a study from France involving 7,923 transplant recipients, the overall prevalence of KS was 0.52% but, in contrast to other studies, the occurrence of KS was more common in liver transplant recipients (1.24%) than in recipients of kidneys (0.45%) or hearts (0.41%) (Farge 1993).

2.2

KSHV Reactivation After Organ Transplantation

2.2.1

Kidney Transplantation

Most of cases of posttransplant KS apparently develop as a result of viral reactivation, because more than 80% of transplant recipients with KS are seropositive for KSHV before undergoing transplantation (Cattani et al. 2001) (Table 2). In an Italian retrospective study, 10 of 11 organ recipients (91%) who developed KS were KSHV seropositive before transplantation (Parravicini et al. 1997). These data are supported by those of another study, which showed that KS developed in three of 21 recipients (14.3%) who were KSHV seropositive before kidney transplantation (Andreoni et al. 2001). It has been shown that among patients who are seropositive for KSHV before undergoing kidney transplantation the risk of posttransplant KS is 23% to 28%, as compared with a risk of 0.7% in patients who are seronegative before receiving a kidney transplant (Cattani et al. 2001; Frances et al. 2000). A French retrospective study performed between 1990 and 1996 showed that 32 patients (8%) had antibodies to KSHV at the time of transplantation and that among these 32 patients, 3 years after transplantation, graft survival was 72% and KS prevalence was 28% (KS incidence: 8.2/yr/100 KSHV+ recipients). Multivariate analysis identified severe bacterial and/or *Pneumocystis carinii* infection (odds ratio: 8.6; $P=0.019$) as a factor associated with KS. This

Table 2 Solid organ transplant recipients with KSHV reactivation

Reference	Type of T.	Country	No. of recipients KSHV positive before T. (%)	No. of KS patients (%)
Cattani et al.	Kidney	Italy	26/175 (15)	6/26 (23)
Frances et al.	Kidney	France	32/400 (8)	9/32 (30)
Andreoni et al.	Kidney and liver	Italy	21/130 (16)	3/21 (14)
Marcelin et al.	Liver	France	3/122 (2.5)	0/3 (0)
Aseni et al.	Liver	Italy	4/459 (0.9)	4/4 (100)
Emond et al.	Heart	France	4/150 (2.7)	1/4 (25)
Sachsenberg et al.	Lung	Switzerland	1 recipient	1

No., number; T., transplantation; KSHV, Kaposi sarcoma-associated herpesvirus; KS, Kaposi sarcoma

suggests that the level of immunosuppression may be an important factor in the development of posttransplant KS.

2.2.2

Other Types of Organ Transplantation

Kaposi sarcoma (KS) has been reported after solid organ transplantation mostly in recipients of renal graft but also in liver, heart, lung, and bone allografts.

Concerning liver transplantation, an Italian study on 130 kidney and liver recipients reported a KSHV prevalence rate of 16.1% before transplantation (Andreoni et al. 2001). In this study, none of the liver transplant recipients developed KS after transplantation. Similarly, among 122 French liver recipients, antibodies to KSHV were detected in sera of three recipients (2.4%) before transplantation, and none of these three recipients developed a KS during the follow-up (Marcelin et al. 2004). However, another Italian study reported the clinical course and outcome of five adult patients who underwent liver transplantation and developed KS (months 9–23 after transplantation) among 459 studied patients. Four of five patients died of KS, surviving 0–6 months after pathological diagnosis, suggesting a high rate of graft involvement and mortality in this setting (Aseni et al. 2001).

In the context of heart transplantation, several cases have been reported. For example, a case report has described a primary effusion lymphoma associated with KSHV infection in a cardiac transplant recipient, who was

probably infected before the graft (Jones et al. 1998). Later, a French study reported a low rate of KS in heart transplant recipients: One of 150 patients (0.67%) who was seropositive for KSHV before transplantation developed iatrogenic KS after heart transplantation. This low rate could be explained by the fact that KSHV is not endemic in France in the general population (prevalence of 2%) (Marcelin et al. 1998).

Very few cases of patients developing KS after lung transplantation have been described. A case report described a KS of the skin, but also of the lung graft. Serological tests showed KSHV seronegativity of the graft donor and KSHV seropositivity of the patient before lung transplantation, suggesting that the latter was already infected before the surgery and that immunosuppression resulted in the development of KS (Huang et al. 2003; Sachsenberg-Studer et al. 1999).

These data suggest that KS occurs predominantly among kidney allograft recipients, but that recipients of other organs are also at risk for KSHV-related disease, even those living in an area in which the virus is not very prevalent.

Finally, substantial morbidity and mortality are associated with a diagnosis of KS among transplant recipients. In a large transplant tumor registry, 143 (40.2%) of 356 patients with KS had visceral involvement, and 61 (17.1%) of 356 had KS listed as their cause of death (Penn 1997). Although the reduction or cessation of immunosuppressive treatment can lead to the complete remission of KS, among patients undergoing remission 65% had graft loss or impaired function, compared with 21% of the overall population of transplant recipients.

2.3

KSHV Contamination After Organ Transplantation

2.3.1

Kidney Transplantation

The first report suggesting some evidence that *de novo* infection from transplantation can occur reported the case of a kidney transplant patient who was KSHV seronegative before transplantation and who seroconverted 13 months after transplantation with onset of Castleman disease (Parravicini et al. 1997). Other KSHV seroconversions after kidney transplantation were identified in Switzerland. In a cohort of 220 transplant recipients, KS developed within 26 months in two of 25 patients (8%) who had developed a primary KSHV infection after kidney transplantation (Regamey et al. 1998). These studies have suggested for the first time that transmission of KSHV can occur by the organ allograft (Table 3). This was further confirmed in other studies, showing rates of KSHV seroconversion after kidney transplantation of 2%

Table 3 Solid organ transplant recipients with KSHV transmission from the donor

Reference	Type of T.	Country	Cases of transmission or rate of seroconversion (%)	No. of KS patients after seroconversion (%)
Luppi et al.	Kidney	Italy	2 patients	2/2
Regamey et al.	Kidney	Switzerland	25/220 (17)	2/25 (8)
Milliancourt et al.	Kidney	France	6/287 (2)	0/6 (0)
Munoz et al.	Kidney, liver and heart	Spain	3/59 (5)	4/70 (5.7)
Emond et al.	Heart	France	1/146 (0.7)	0/1 (0)
Marcelin et al.	Liver	France	4/122 (0.3)	2/4 (50)

No., number; T., transplantation; KS, Kaposi's sarcoma

and 5% in France and Spain, respectively (Milliancourt et al. 2001; Munoz et al. 2002). Moreover, a primary KSHV infection has been described in two patients after both received kidneys from a seropositive cadaver donor. One of the patients developed disseminated KS, and the other developed an acute syndrome characterized by fever with plasmacytosis followed by bone marrow failure and ultimately died of renal and cardiac failure (Luppi et al. 2000). In a more recent study, sex and genetics markers specific to the donor were detected in neoplastic KSHV-infected cells in KS lesions isolated from renal transplant recipients. This finding suggests that not only KSHV but also KS progenitor cells may be seeded after solid organ transplantation, survive in the recipients, and undergo neoplastic transformation and progression (Barozzi et al. 2003).

2.3.2

Other Types of Organ Transplantation

Similar low rates of KSHV seroconversions have been reported after heart and liver transplantations. Among a group of 150 patients awaiting heart transplants only one of them seroconverted for KSHV antibodies after transplantation, without developing KS (Emond et al. 2002). In the context of liver transplantation, among 122 liver donors and their respective recipients, four primary KSHV infections were detected among KSHV-seronegative recipients who received a liver from a KSHV-positive donor. Among these four recipients, two particularly immunosuppressed patients developed symptomatic

diseases and died a few months after transplantation, harboring disseminated KS and KSHV-positive lymphoproliferation. In these two patients, KSHV DNA genome sequences were detectable in peripheral blood mononuclear cells (PBMCs) and other tissues with high viremia levels before and at the beginning of KSHV-related diseases (Marcelin et al. 2004). This suggests that KSHV serological assay and PCR should be used in combination to monitor recipients having received an organ from a KSHV-positive donor (Pellet et al. 2002).

In conclusion, even if KSHV primary infections after organ transplantation seem to be rare events, this can lead in some cases to a fatal outcome.

2.3.3

KSHV Serology in the Context of Seroconversion

KSHV is not ubiquitous in the general healthy population, at least in United States and in Northern Europe, and therefore serology is more useful than other methods in detecting primary infections. Current serological assays are immunofluorescence assays (IFA) using KSHV-infected B cell lines and enzyme-linked immunosorbent assays (EIA) that use isolated KSHV proteins or peptides. Both types of assays can detect antibodies directed against viral proteins expressed during a lytic, replicative cycle or during latency. KSHV antigens that are targeted by the serological response come in two broad categories: latent and lytic. The major latent antigen is latent nuclear antigen 1, which is encoded by open reading frame (ORF)-73. The major lytic antigens are ORF65 (a capsid protein) and K8.1 (a membrane-associated glycoprotein). Although there is some debate regarding which assay is most accurate, the IFA using lytic cycle antigens appears to be the most sensitive assay (Spira et al. 2000; Zhu et al. 1999). Indeed, the rate of seroconversion could be underestimated with the use of only IFA latent assays. This was highlighted in a study in which no seroconversion event was observed with latent IFA assay even in two liver recipients who developed documented symptomatic primary KSHV infection (Marcelin et al. 2004). However, with lytic IFA assay, all the patients who received grafts from KSHV-infected donors seroconverted for KSHV. This can be related to the fact that antibodies targeted against lytic antigens appeared before antibodies directed against latent antigens, which is well known for EBV. Thus the use of a combination of both latent and lytic assays to evaluate seropositivity should avoid the possibility of false negative results and provide an exact evaluation of the rate of seroconversion after transplantation.

3 Risk Factors Associated with Posttransplant KS Development in Solid Organ Transplant Recipients

Almost all solid organ transplant patients with KS have been found to be seropositive against KSHV, with doubtful exceptions (Briz et al. 1998; Demirag et al. 1998; Farge et al. 1999; Qunibi et al. 1998; Regamey et al. 1998). However, several factors may influence the risk of developing the disease in patients with prior infection, including the ethnic origin of the patients, the seroprevalence rate in their geographic origin, sex, age, and degree of immunosuppression (Calabro et al. 1998; Gao et al. 1996b; Lennette et al. 1996; Penn 1997). Incidence of posttransplant KS is higher in countries or ethnic groups with high seroprevalence against KSHV (Farge et al. 1999). Seroprevalence of KSHV antibodies varies from 2% to 50%. KSHV antibodies have been found in 2% to 14% of studied subjects in Europe (Belec et al. 1998; Frances et al. 1999; Marcelin et al. 1998; Regamey et al. 1998; Whitby et al. 1998) and in 50% of patients with renal transplants in a study in Texas (Hudnall et al. 1998). KSHV antibodies exceed 50% in some African countries (Gao et al. 1996a; Mayama et al. 1998; Plancoulaine et al. 2000).

KS appears in younger patients compared with other posttransplant malignancies, with a median age around 35–40 years (Euvrard et al. 2003). Some pediatric cases have also been reported (Fournet et al. 1992; Penn 1994). Male patients predominate in most series and types of KS, with male-to-female ratios around 2.5–4.0 to 1 in solid organ transplant recipients (Ecdler et al. 1998; Montagnino et al. 1994). Genetic predisposition related to the presence of some HLA antigens in KS patients is no longer sustained (Brunson et al. 1990; Qunibi et al. 1988; Sungur et al. 1995; Zong et al. 1999).

Finally, the degree of immunosuppression is a critical factor. It has been demonstrated that renal transplant recipients have a higher incidence of KS than nongrafted patients on maintenance dialysis (Montagnino et al. 1996; Qunibi et al. 1998). It is not clear, however, how the use of different immunosuppressive agents influences the development of KS. There is no definitive evidence of a higher rate of KS related to the use of a specific drug. In vitro studies have shown that immunosuppressive agents such as hydrocortisone and cyclosporine do not activate the lytic cycle of KSHV and do not modify the cell survival, thus promoting cancer progression by a direct cellular effect (Marcelin et al. 2001). However, administration of more than 10 corticosteroid pulses has been claimed to correlate with a higher incidence of the disease (Montagnino et al. 1996). In the same way, a severe KSHV-related disease was reported in two recipients who received a liver from a KSHV positive donor and who had a stronger immunosuppression compared to the other recipi-

ents, which was induced by higher tacrolimus trough levels and use of steroid boluses (Marcelin et al. 2004). The relative influence of different immunosuppressive agents is not totally clear (Cockburn and Krupp 1989; Eberhard et al. 1999; Farge et al. 1999; Hiesse et al. 1995; Rezeig et al. 1997). Poor donor-recipient matching has not been found to be a risk factor for KS (28).

4

KSHV Screening and Monitoring in the Context of Solid Organ Transplant Recipients

4.1

KSHV Screening

In the light of seroepidemiological studies showing high prevalence of KS within the studied populations of KSHV-positive transplant recipients, the possibility of transmission of the virus through the graft leading in some cases to fatal outcome, and finally the low graft survival, it is tempting to raise the question of the benefit of systematic screening of KSHV serological features before transplantation. Although studies have indicated that KSHV transmission by the organ allograft is uncommon, serological screening of donors for KSHV infection may be necessary, especially in geographic areas with high seroprevalence of KSHV infection. Although most cases of KS in transplant recipients are the result of KSHV reactivation, avoiding matches between KSHV-positive donors and KSHV-negative recipients would prevent some occurrence of KS. Although the current evidence is insufficient to recommend against performing a transplant on the basis of KSHV mismatches, cost/benefit analyses for strategies are needed that account for transplant type (renal, liver, etc.) and regional prevalence of KSHV infection. Regardless of whether donor/recipient seromatching proves to be beneficial, the use of posttransplantation KSHV diagnostics to identify recipients at high risk is likely to provide a benefit because of heightened surveillance and the possible prophylaxis or treatment for KS.

4.2

KSHV Monitoring

Knowledge of the KSHV infection status of graft recipients could help in identifying individuals at high risk for posttransplantation KS and in carefully monitoring them clinically and biologically. Moreover, the systematic screening of donors for KSHV, not to exclude the graft but rather to know the KSHV

serological status and to monitor the recipient adequately, could be helpful. At the moment, routine virological assessment of organ donors includes, at least in most countries in Europe, HIV, HCV, and HTLV1 serology, where positivity excludes from organ donation. HBV testing comprises HBsAg, anti-HBs, and anti-HBc detection. Although HBsAg positivity excludes from organ donation, anti-HBc-positive donors may be used under certain life-threatening circumstances, even if their infectivity is clearly established (Roque-Afonso et al. 2002). However, HBV prophylaxis with antiviral agents or immunoglobulins may prevent HBV reactivation from the liver graft. A similar strategy could be developed for KSHV in the context of organ transplantation, to avoid the occurrence of severe KSHV-related disease, combining use of serological assays in donors and recipients and molecular detection of KSHV DNA sequences in recipients of positive donors.

This strategy could allow the use of several potential interventions suggested by our current understanding of the relationship of the virus to disease. The utility and efficacy of such interventions will need careful clinical evaluation and refinement as part of the process of developing a new standard of care for immunocompromised patients who are infected with KSHV. For example, reduction or discontinuation of immunosuppressive drugs is usually associated with regression of lesions, and this strategy could be proposed preventively in cases of high risk of development of KSHV-related disease, for example, in patients harboring a positivation and/or increase of KSHV viral load. Moreover, the value of an antiviral prophylaxis to lower the risk of KS needs to be explored. There is evidence to suggest that prophylaxis with antiherpes drugs, such as foscarnet, ganciclovir, and cidofovir, protects against the development of KS. Although no drugs are currently licensed for KSHV therapy, in a large clinical trial Martin et al. showed that, among HIV-infected patients treated for cytomegalovirus retinitis, both oral and intravenous ganciclovir led to significant decreases in the incidence of KS (Martin et al. 1999). The benefit in term of prophylaxis of some drugs, such as val-ganciclovir, which is used to prevent cytomegalovirus infection in transplant recipients, should be evaluated in KSHV-infected organ transplant recipients. Two recent studies reported remission of multicentric Castlemans disease with anti-CD20 monoclonal antibody infusion correlating with reduction of KSHV DNA viral load in PBMCs (Corbellino et al. 2001; Marcelin et al. 2003). Thus the use of anti-CD20 monoclonal antibody could be evaluated in cases of documented primary KSHV infection with the aim of decreasing the risk of severe KSHV-related disease.

4.3

Limitations

However, before recommending systematic screening of KSHV in candidates for transplantation and organ donors, several questions remain to be resolved.

First, there is a lack of large prospective studies evaluating the prevalence of KSHV in organ donors and recipients and the exact risk of posttransplant KS and the related rate of morbidity and mortality. Such a prospective study is ongoing in France including all the transplantation centers. Preliminary data have been presented and showed that KSHV prevalence among kidney donors and recipients was 1.05% (40/3,819 cases) and 2.87% (165/5,749 cases), respectively. To date, 21/165 (13%) of patients developed posttransplant KS among patients KSHV seropositive before transplantation (median follow-up of 608 days; range 0–1,377). The utility of the measurement of KSHV viremia as a predictor of the development of KS will be evaluated. Indeed, periodic monitoring of the KSHV load in PBMCs could help to identify the subset of patients who are at the highest risk for KS, and early detection may be important, because KS appears to be more likely to improve if it is treated at an early stage. This has been suggested by studies that found a lower KS tumor burden to be a predictor of a complete KS response to HAART in HIV-infected patients (Dupin et al. 1999) and a predictor of complete KS remission in organ transplant recipients who stop taking their immunosuppressive therapy (Shepherd et al. 1997).

Second, there is still some debate regarding which KSHV serological assay is most accurate. Because no “gold standard” for determining positivity for KSHV antibodies is yet available and there is a lack of standardization and commercial availability, it is difficult to recommend specific assays or formats for clinical use. However, the combination of a lytic and a latent nuclear antigen IFA should provide the best combination of sensitivity and specificity (Ablashi et al. 2002; Schatz et al. 2001). The use of multiple assays is particularly important in determining prevalence in asymptomatic populations (Biggar et al. 2003). The performance of such combined assays indicates that they may be used for the clinical management of individuals at risk of developing KSHV-associated tumors such as allograft recipients. Moreover, most of these assays are manual and are not adapted to emergency situations, as no EIA simultaneously had sensitivity and specificity above 90% (Engels et al. 2000). Many studies have suggested the potential utility of screening of KSHV antibodies among organ donors and recipients. However, to date the results of these studies have argued in favor of KSHV screening, even in low-KSHV infection prevalence countries, not to exclude the graft but to obtain KSHV status information in order to have the opportunity to monitor, clinically and biologically, patients at risk for KSHV-related disease development. The de-

tection of KSHV antibodies could be done in the days after the transplantation and the results transmitted to physicians retrospectively.

In conclusion, the question of screening donors and recipients for KSHV, even in low-KSHV infection prevalence countries, is still debated, and prospective studies are needed to evaluate the benefit of pre- and posttransplantation strategies.

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Kaposi Sarcoma-Associated Herpesvirus and Other Viruses in Human Lymphomagenesis

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1	Introduction	264
2	KSHV	265
2.1	Association of KSHV and Malignant Lymphomas	265
2.2	Role of KSHV in PEL	268
2.3	Molecular Mechanisms of KSHV-Induced Lymphomagenesis	268
2.3.1	Latent Viral Proteins	269
2.3.2	Lytic Viral Proteins	271
2.4	Animal Models for KSHV-Associated Lymphomas	273
2.5	Current and Future Therapeutic Approaches for KSHV-Associated Lymphomas	273
3	Other Lymphomagenic Viruses	274
3.1	Epstein-Barr Virus	274
3.2	Human T-Cell Lymphotropic Virus-1 and Adult T-Cell Leukemia/Lymphoma	276
4	Conclusions	278
	References	279

Abstract Kaposi sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV-8), is associated with a specific subset of lymphoproliferative disorders. These include two main categories. The first is primary effusion lymphomas and related solid variants. The second is multicentric Castleman disease, from which KSHV-positive plasmablastic lymphomas can arise. KSHV contributes to lymphomagenesis by subverting the host cell molecular signaling machinery to deregulate cell growth and survival. KSHV expresses a selected set of genes in the lymphoma cells, encoding viral proteins that play important roles in KSHV lymphomagenesis. Deregulation of the NF- κ B pathway is an important strategy used by KSHV to promote

lymphoma cell survival, and the viral protein vFLIP is essential for this process. Two other viruses that are well documented to be causally associated with lymphoid neoplasia in humans are Epstein-Barr virus (EBV/HHV-4) and human T-cell lymphotropic virus (HTLV-1). Both of these are similar to KSHV in their use of viral proteins to promote cell survival by deregulating the NF- κ B pathway. Here we review the basic information and recent developments that have contributed to our knowledge of lymphomas caused by KSHV and other viruses. The understanding of the mechanisms of viral lymphomagenesis should lead to the identification of novel therapeutic targets and to the development of rationally designed therapies.

1

Introduction

In the early twentieth century Ellerman and Bang made the first observation that a viral infection could lead to the development of leukemia (Ellerman and Bang 1908). Since that time, many associations between viruses and leukemia/lymphoma have been shown in a variety of animals, but only a few firmly have been demonstrated in humans. Nowadays, in order to consider that a viral entity is causally associated with a human cancer, it must fulfill the criteria of four so-called Koch-like postulates (Zur Hauzen 1999): (1) epidemiologic evidence that the virus represents a risk factor for the development of the tumor, (2) consistent presence or persistence of the viral genome in the tumor cells, (3) documented oncogenic effects of viral infection or introduction of viral genes to cells, and (4) demonstration that the malignant phenotype of tumor cells depends on functions conferred by the viral genomes. Based on these rigorous criteria only three human viruses are clearly directly associated with lymphoid neoplasia: Epstein-Barr virus [EBV; formally called human herpesvirus 4 (HHV-4)], Kaposi sarcoma-associated herpesvirus (KSHV; human herpesvirus 8 (HHV-8)], and human T cell lymphotropic virus 1 (HTLV-1).

Although the prevalence of lymphomagenic viruses in the normal population is quite high (>95% of adults for EBV), only a small number of infected immunocompetent people ever develop the virus-induced malignancy and do so only after a long latency period. This observation reflects the multi-step nature of oncogenesis, with the viral infection representing only one of these steps. In the following sections, the current understanding and recent developments concerning the pathobiology of KSHV and its role in lymphomagenesis are reviewed. We also briefly review the similarities between KSHV and two other human lymphomagenic viruses, EBV and HTLV-1.

2 KSHV

2.1 Association of KSHV and Malignant Lymphomas

KSHV is found invariably in Kaposi sarcoma, and compelling evidence suggests that it is an etiologic agent for this disease (Chang et al. 1994; Moore and Chang 1995). KSHV is also present in several lymphoproliferative disorders. Shortly after its first discovery, the presence of the KSHV genome was recognized in a subset of lymphomas in HIV-positive patients, all of which occurred as lymphomatous effusions in body cavities and were first called body cavity-based lymphomas and subsequently primary effusion lymphomas (PELs) (Cesarman et al. 1995a; Nador et al. 1996). Although they are more common in HIV-positive males, PELs also occur in HIV-negative men and women (Nador et al. 1995; Said et al. 1996). PELs contain many KSHV genomes, ranging between 40 and 80 copies per cell, which contrasts with an average of one copy per cell in KS lesions (Fig. 1A). The presence of KSHV in this subset of lymphomas allowed the development of cell lines that have been used as a tool for its propagation and for serologic assays (Arvanitakis et al. 1996; Boshoff et al. 1998; Cesarman et al. 1995b; Renne et al. 1996). Although these cell lines contain mostly latent virus, expressing a restricted number of viral genes, they can be induced to undergo lytic reactivation with phorbol esters or butyrate, thus producing infectious virus (Fig. 1A). Purified virus from PEL cell lines has been used to demonstrate its ability to infect B cells (Mesri et al. 1996), endothelial cells (Cannon et al. 2000; Flore et al. 1998; Moses et al. 1999; Panyutich et al. 1998), and cell lines from a variety of derivations (Bechtel et al. 2003).

Because some KSHV-negative lymphomas, such as Burkitt lymphoma (BL), can involve body cavities as lymphomatous effusions, and KSHV-positive lymphomas can present as solid-tissue masses, diagnostic criteria for PEL have been proposed (Cesarman and Knowles 1999; Nador et al. 1996). These criteria include immunoblastic-anaplastic large-cell morphology (Fig. 2), null-cell phenotype (including the lack of B cell-associated antigen and immunoglobulin expression in most cases), and B-cell genotype. The diagnosis of a KSHV-associated malignancy now can be easily confirmed by immunohistochemistry, as antibodies to viral proteins are commercially available (Fig. 2). The expression of CD138/syndecan-1 (Gaidano et al. 1997) and hypermutation of the immunoglobulin genes (Fais et al. 1999; Matolcsy et al. 1998) suggest that PELs are at a postgerminal center stage of B-cell differentiation and close to the plasma cell stage. This assumption was more recently confirmed by gene expression profiling of PEL (Jenner et al. 2003;

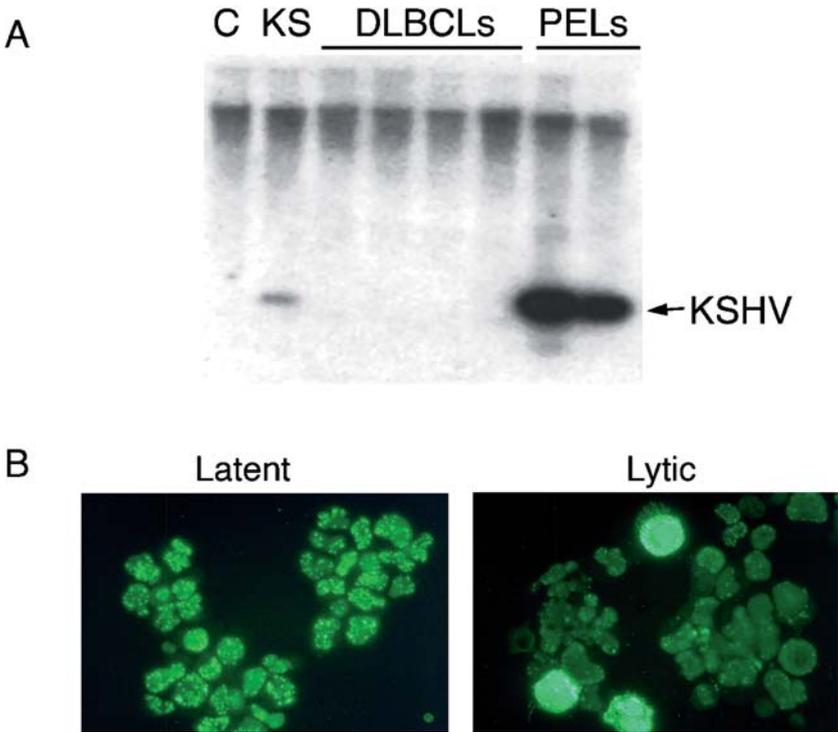


Fig. 1 Detection of the KSHV genome and latent and lytic antigens in PEL cell. Lines. **A** Southern blot hybridization using a probe from the KSHV genome detected a strong band in *Bam*H 1-digested DNA extracted from two cases of PEL and a weaker band in Kaposi sarcoma, but not in a panel of diffuse large B cell lymphoma (DLBCLs) or in the HL60 cell lines used as the negative control (C). **B** Immunofluorescence assays were performed on the BC-3 PEL cell line, which was either untreated (*left*) or treated with tetradecanoyl phorbol acetate (TPA; *right*), using serum from a patient with KS as primary antibody. Positivity in latent cells is seen by a speckled nuclear pattern due to recognition of LANA-1 by the antiserum. TPA induces lytic reactivation in three cells in this field, which is visualized as the recognition of numerous nuclear and cellular proteins

Klein et al. 2003), where PEL resembled plasma cells and had a profile between multiple myeloma and EBV-associated immunoblastic lymphoma.

Lymphomas containing KSHV can also occur at presentation as solid tissue masses, similar to other AIDS-related non-Hodgkin lymphomas. Although some of these lymphomas subsequently develop an effusion, others apparently do not. They usually present as solid extranodal lymphomas and are diagnosed as diffuse large-cell, immunoblastic, or anaplastic large-cell lym-

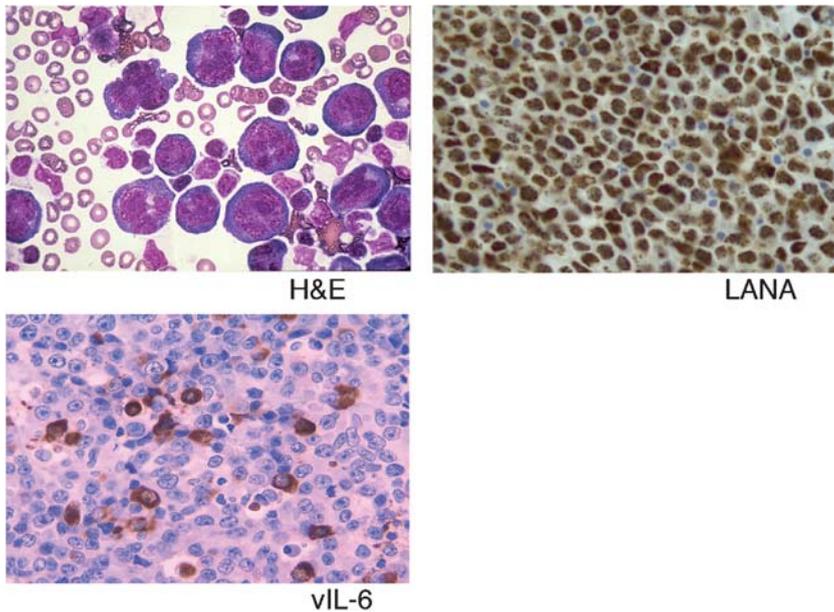


Fig. 2 Morphology and immunohistochemistry for KSHV of primary effusion lymphoma. An air-dried cytocentrifuge preparation of a KSHV-positive primary effusion lymphoma was stained with Wright-Giemsa (*left panel*). The tumor cells in this image are considerably larger than normal benign lymphocytes and monocytes. The cells display significant polymorphism and possess moderately abundant basophilic cytoplasm. A prominent, clear perinuclear Golgi zone can be appreciated in several cells. The nuclei vary from large and round to highly irregular, multilobated, and pleomorphic and often contain one or more prominent nucleoli (original magnification $\times 1,000$). Immunohistochemical staining with the following antibodies is shown: LANA (ORF 73), a monoclonal rat antibody was used, showing brown speckled nuclear positivity in all the neoplastic cells; vIL-6, immunohistochemical staining with a polyclonal rabbit antiserum shows abundant cytoplasmic expression (*brown*) in many lymphoma cells (original magnification $\times 400$)

phomas, in which the presence of KSHV in practically all the lymphoma cells can be demonstrated by immunohistochemistry or molecular techniques (Carbone et al. 2005; Chadburn et al. 2004; DeLoose et al. 2005; Engels et al. 2003). Most of these are immunoblastic in appearance and have a high mitotic rate and variable amounts of apoptotic debris. These lymphomas appear to fall in the spectrum of PEL, as they frequently lack expression of B-cell antigens and immunoglobulin, they have a similar morphology, and the majority are coinfecting with EBV.

Plasmablastic lymphomas, associated with multicentric Castleman disease, have also been described in HIV-positive patients (Dupin et al. 2000). Although these plasmablastic lymphomas are KSHV positive, they differ from PEL in a number of ways. Plasmablastic lymphomas are EBV negative, do not contain mutations in the Ig genes, and are thought to arise from naive IgM lambda-expressing B cells rather than terminally differentiated B cells (Du et al. 2001). In addition, KSHV has been documented in germinotropic lymphoproliferative disorders in HIV-negative patients (Du et al. 2002), suggesting that this virus is present in a heterogeneous but distinct group of lymphoproliferative diseases and may be more common than previously thought.

2.2

Role of KSHV in PEL

The almost invariable presence of KSHV in PELs suggests that it is necessary for their development. However, PELs are rare tumors, even in populations with high KSHV seroprevalence, accounting for about 3% of AIDS-related lymphomas and 0.4% of all AIDS-unrelated diffuse large-cell NHLs (Carbone et al. 1996). Therefore, it is evident that KSHV infection represents only one of several events involved in the development of PEL. Another cofactor appears to be EBV, because most PELs contain both viral genomes. The majority of PELs in vivo, as well as in culture, are latently infected with both viruses, but there is a small proportion of cells in which EBV and/or KSHV productive infection takes place. Analysis of the pattern of EBV gene expression in PELs revealed that only EBNA1 was expressed, corresponding to Latency I (Horenstein et al. 1997; Szekely et al. 1998). In addition, PELs lack structural alterations in most cell-transforming genes frequently involved in lymphomagenesis, with the possible exception of mutation in the regulatory region of Bcl-6 (Carbone et al. 1998; Nador et al. 1996). Although there are many cytogenetic abnormalities, reflecting genomic instability, none of these is consistent in all PELs. KSHV expresses a small number of viral proteins in PEL cells, but practically all of these have been documented to have oncogenic potential. These observations suggest that KSHV plays a transforming role in PELs.

2.3

Molecular Mechanisms of KSHV-Induced Lymphomagenesis

The majority of PELs are latently infected by KSHV. Latency allows the virus to remain in the infected cell, ensuring that the cell survives and is not recognized as infected by the host immune system. On initial infection, KSHV produces viral proteins that inhibit innate antiviral responses, and subsequently during latency it produces a protein that ensures maintenance of viral

DNA in the form of extrachromosomal circles, called episomes, in dividing cells. It also produces proteins during latency that promote survival of the infected cells. Because promotion of cell survival is a main feature of cancer, it is not surprising that KSHV is associated with malignancies. In fact, given the number of proteins produced by KSHV with oncogenic potential, the surprise comes from the relative rarity of KSHV-associated cancers.

2.3.1

Latent Viral Proteins

Five major KSHV proteins have been confirmed to be produced in latently infected lymphoma cells. They are called LANA-1 (also LANA or LNA), vCyclin (vCYC), vFLIP, vIL-6, and LANA-2 (or vIRF-3). Of these, LANA-1, vCYC, and vFLIP are transcribed from the same region into a single tricistronic mRNA, which gets alternatively spliced into a transcript containing LANA-1 and another encoding for vCYC and vFLIP, the expression of which comes from an internal ribosomal entry site (IRES) (Bielecki and Talbot 2001; Low et al. 2001; Talbot et al. 1999). Other proteins have also been reported to be expressed during latency (like K15 and the Kaposins), but these data are either controversial or not confirmed at the protein level. All of these genes bear potential to participate in lymphomagenesis and the maintenance of the malignant phenotype by affecting cellular survival and/or proliferation. Their function is illustrated in Fig. 3 and can be summarized as follows:

1. LANA-1: This protein, essential for episome maintenance (Ballestas et al. 1999), has been shown to have the ability to transform cells (Radkov et al. 2000) and induce lymphoid tumors in transgenic mice (Fakhari et al. 2006). LANA-1 has the ability to affect several pathways that are involved in tumorigenesis: It can bind and inactivate the retinoblastoma (Rb) protein (Radkov et al. 2000) and similarly bind and inactivate p53 (Friborg et al. 1999), protecting cells from apoptosis and inducing genetic instability (Si and Robertson 2006). LANA-1 can also bind and inactivate GSK-3 β , leading to activation of the β -catenin pathway involved in solid tumors (Fujimuro and Hayward 2004). It has also been shown to have transcriptional effects, both positive and negative, on a variety of cellular and viral genes.
2. vCYC: This protein is a functional cyclin that can associate with CDK6 and induce phosphorylation of Rb protein and overcome Rb-mediated cell cycle arrest (Godden-Kent et al. 1997; Li et al. 1997). vCYC differs from the cellular cyclin D in its ability to induce degradation of the CDK inhibitor p27Kip when complexed with CDK6 (Ellis et al. 1999; Mann

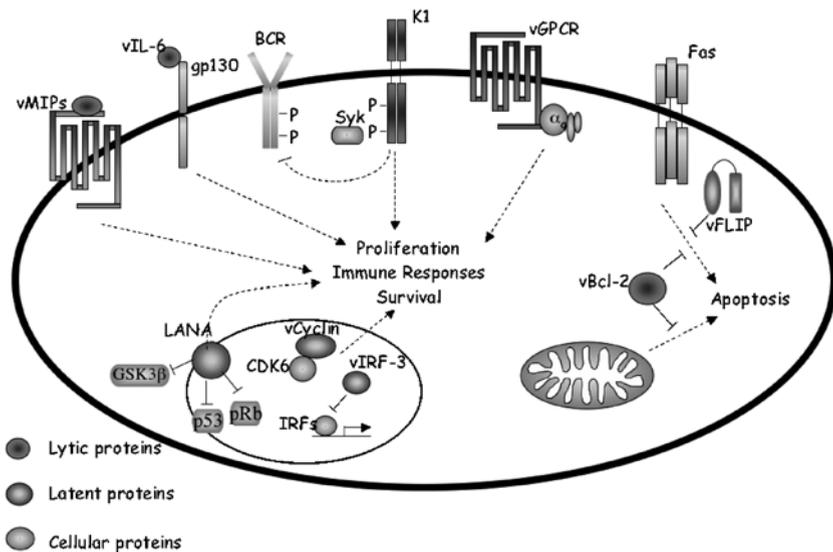


Fig. 3 Representative KSHV-encoded proteins that can affect cellular proliferation, survival, differentiation, and/or angiogenesis. KSHV contains several open reading frames encoding homologues to cellular proteins involved in vital proliferative and survival functions. Among these are (1) secreted autocrine/paracrine factors (vIL-6 and vMIPs); (2) transmembrane signaling molecules (vGPCR and K1); (3) transcriptional regulators (vIRFs and LANA); (4) a cell cycle regulator (vCYC); (5) tumor suppressor gene-binding activity (LANA) and (6) apoptosis inhibitors (vBCL-2 and vFLIP). Those genes clearly demonstrated to be expressed in latently infected cells of Kaposi's sarcoma are illustrated in *ovals*. (Reprinted from Cesarman and Mesri (2006))

et al. 1999). Therefore, vCYC is likely to modulate the cell cycle in PEL cells, avoiding normal regulatory checkpoints. Transgenic mice with vCYC develop lymphomas, but only in the absence of p53, and a model has been proposed whereby vCYC induces genome instability and loss of p53 subsequently allows expansion of tumorigenic clones (Verschuren et al. 2004, 2002).

3. vFLIP (K13): This protein is homologous to cFLIP, which is an inhibitor of the proapoptotic molecule FLICE/Caspase-8 (Thome et al. 1997). vFLIP can bind to TRAFs and localize to the IKK complex, activating NF- κ B through both the classic and alternative pathways (Chaudhary et al. 1999; Field et al. 2003; Guasparri et al. 2006; Matta and Chaudhary 2004), and through this mechanism induce expression of antiapoptotic genes and protect cells from Fas-induced cell death *in vitro* and *in vivo* (Djerbi et al.

- 1999; Guasparri et al. 2004). vFLIP protects PEL cells from spontaneous apoptosis and is the first viral protein shown to be essential for tumor cell survival *in vitro* and *in vivo* (Godfrey et al. 2005; Guasparri et al. 2004).
4. LANA-2 (vIRF-3): Although this protein is expressed by only rare cells in KS lesions, indicating a lytic expression pattern in endothelial cells, it is expressed by most PEL cells, being a latent protein in lymphomas (Rivas et al. 2001). This protein was shown to potently inhibit p53 in reporter assays (Rivas et al. 2001) and thus may be involved in deregulation of apoptosis. Although LANA-2 is not a DNA-binding protein, it is recruited to the interferon promoters via its interaction with cellular IRF-3 and IRF-7 and stimulates their transcriptional activity (Lubyova et al. 2004).
 5. vIL-6: This is sometimes considered to be a lytic gene because its expression is increased on lytic reactivation. However, it is expressed by a variable but significant proportion of latently infected PEL cells. Considering the fact that this protein can be secreted, it may affect other tumor cells that do not express it and play a role in their proliferation. vIL-6 differs from cellular IL-6 in that it is selectively glycosylated and can bind the gp130 receptor in the absence of the high-affinity IL-6 receptor to activate IL-6-responsive genes and promote B-cell survival (Molden et al. 1997; Moore et al. 1996; Nicholas et al. 1997). vIL-6 has been shown to induce expression of vascular endothelial growth factor (VEGF) (Aoki et al. 1999), which in turn can induce effusion development (Aoki and Tosato 1999; Liu et al. 2001), therefore being involved in the pathogenesis of PEL.

2.3.2

Lytic Viral Proteins

Although mostly latent genes are expressed in PELs, some lytic genes are expressed to some extent in a minority of cells, and several of these have oncogenic potential. In addition, patterns of KSHV gene expression *in de novo* infection and during initial steps of lymphomagenesis are not known. These lytic genes encode for some functionally interesting proteins. The viral interferon regulatory factor 1 (K9, vIRF-1) (Li et al. 1998), the viral G protein-coupled receptor (ORF 74, vGPCR) (Bais et al. 1998), and ORF K1 (Lee et al. 1998b) can transform rodent cells and/or cause tumors in animal models. Three transmembrane proteins are potentially important in PEL lymphomagenesis because they can trigger signaling cascades relevant for B- and T-cell growth. These are:

1. **vGPCR:** This is a constitutively active G protein-coupled receptor able to trigger the mitogen-activated protein (MAP) kinase signaling cascades and induce secretion of VEGF (Bais et al. 1998). MAP kinase cascades such as those triggered by vGPCR are activated by inflammatory cytokines and mitogens. VEGF is an angiogenic and vascular permeability factor that could contribute to the effusion phenotype (Aoki and Tosato 1999). In PEL cells, vGPCR can activate a variety of signaling cascades activating the transcription factors AP-1, NF- κ B, CREB, and NFAT (Cannon et al. 2003; Cannon and Cesarman 2004). vGPCR can induce cell cycle arrest that is mediated by p21 (Cannon et al. 2006). Cell cycle arrest can be induced by other KSHV lytic genes (Wu et al. 2002) and is common during the lytic phase of other viruses including EBV (Rodriguez et al. 2001a, 2001b). However, in the case of vGPCR, this induction of p21 results in inhibition of Cdk2 activity that can delay, and perhaps abort, lytic replication. This ability of vGPCR to inhibit viral replication may explain how this lytic gene product may still have oncogenic effects by inducing the expression of multiple inflammatory and angiogenic factors.
2. **K1:** This protein has an ITAM motif that can activate cytoplasmic tyrosine kinases and mimic signaling by the B-cell antigen receptor (Lee et al. 1998a). K1 can activate the MAP kinase and NF- κ B signaling pathways and has potential antiapoptotic functions (Brinkmann et al. 2003; Sharp et al. 2002). This viral protein has also been shown to activate the Akt and Src kinases (Prakash et al. 2005; Tomlinson and Damania 2004). Therefore, like vGPCR, it appears to have very broad signaling capabilities.
3. **K15:** This open reading frame encodes a protein with complex splicing and multiple transmembrane motifs, structurally resembling EBV LMP-2A. It has SH2 and SH3 motifs, and also a TRAF binding motif, and can also activate signaling cascades that include NF- κ B and MAP kinase (Brinkmann et al. 2003). Also like EBV LMP-2A, K15 can inhibit B cell receptor signaling (Choi et al. 2000). K15 protein has been found to be present in most latently infected cells by one study (Sharp et al. 2002), but the RNA expression data are controversial, and most studies argue for lytic expression. K15 has been proposed to have antiapoptotic functions (Sharp et al., 2002).

Other interesting molecules that are expressed during lytic replication and have a potential role in PEL pathogenesis are vIRF1, which can inhibit interferon-induced transcriptional activation (Li et al. 1998; Zimring et al. 1998), and vBCL-2 (ORF16), a homologue of cellular BCL-2, which is a cellular oncogene involved in the development of follicular lymphomas. vBCL-2

can block apoptosis as efficiently as cellular Bcl-2, Bcl-xL, or the EBV Bcl-2 homologue, BHRF1 (Cheng et al. 1997). Interestingly, the KSHV Bcl-2 cannot homodimerize or heterodimerize with other Bcl-2 family members, suggesting that it may have evolved to escape any negative regulatory effects of the cellular Bax and Bak proteins.

2.4

Animal Models for KSHV-Associated Lymphomas

Animal models are useful tools to reproduce human disease under controlled experimental conditions in order to study the disease pathobiology and be able to test the effectiveness of drugs. It was first found that PELs injected into SCID/NOD mice developed highly angiogenic immunoblastic lymphomas (Picchio et al. 1997). In efforts to more accurately reproduce PEL disease presentation, PEL cells were injected intravenously (i.v.) and intraperitoneally (i.p.) into NOD/SCID mice (Boshoff et al. 1998; Said et al. 1997). Intravenous injections of PEL cell led to organ infiltration but did not lead to formation of tumors. On the other hand, i.p. injection of PEL led to the development of ascites and diffuse infiltration of organs, without solid lymphoma formation, resembling the diffuse nature of human PEL (Boshoff 1998). This model has been very useful for the preclinical testing of anti-PEL therapeutic approaches (Keller et al. 2006; Wu et al., 2005) (see Sect. 2.5) and for studies on PEL pathobiology. PEL cells are able to secrete VEGF, which is not only angiogenic but also a powerful vascular permeability factor. Thus secreted VEGF could play a role in the effusion phenotype of PELs. Indeed, it was found that anti-VEGF blocking antibodies were able to prevent the formation of PEL ascites, indicating that the VEGF-VEGF receptor axis plays a major role in effusion formation (Aoki and Tosato 1999). Thus it appears that observations made in PELs growing in vivo are able to recapitulate PEL cell-host interactions that may affect viral and host gene expression. This was recently demonstrated by Staudt et al. (Staudt et al. 2004), who found that PEL cells injected in matrigel generated well-organized angiogenic tumors formed by KSHV-infected PEL cells, displaying a pattern of lytic and latent KSHV gene expression that was quite different from the pattern found in PEL cells growing in culture.

2.5

Current and Future Therapeutic Approaches for KSHV-Associated Lymphomas

Survival of PEL patients with conventional chemotherapy is dismal, so it is important to develop novel therapeutic approaches. A promising cellular target is the transcription factor NF- κ B, which is constitutively active in PEL cells,

and pharmacologic inhibition with Bay 11-7082 leads to tumor cell apoptosis (Keller et al. 2000). This compound also resulted in improved survival and cures in a mouse model of PEL as well as a model of EBV-lymphoma (Keller et al. 2006), indicating that NF- κ B inhibition is a viable approach for the treatment of lymphomas associated with herpesviral infections. Although this particular inhibitor is not being pursued with therapeutic intent, other compounds such as the proteasomal inhibitor Bortezomib (Velcade) affect NF- κ B and may be useful for the treatment of PEL. A promising new approach that also targets the NF- κ B pathway is the combination of AZT, a nucleoside analog that also functions as an NF- κ B inhibitor, and IFN- α , an inducer of apoptosis. This combination leads to apoptosis in HHV-8+/EBV-PEL cells in culture, by induction of a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated suicide program. This approach has been proven effective in one reported patient with PEL and has the advantage of using available and clinically approved agents (Ghosh et al. 2003). Recent experiments in the PEL ascites animal model show that this treatment was effective in increasing the mean survival time in mice (Wu et al. 2005). This suggests that induction of apoptosis in PEL cells may be exploited as an effective, relatively nontoxic therapy targeting KSHV-infected PELs.

Although cellular targets such as NF- κ B have the advantage that they are being developed at a rapid pace for other diseases, in particular other cancers and inflammation, viral targets would have the added advantage of specificity and low toxicity. KSHV encodes several genes that can activate NF- κ B, but only vFLIP is largely responsible for the constitutive NF- κ B activity in PEL cells. Elimination of vFLIP results in marked reduction of NF- κ B, decreased expression of NF- κ B-regulated genes and cellular apoptosis (Guasparri et al. 2004). Therefore, vFLIP functions as a viral oncogene in KSHV-associated lymphomas and is a promising therapeutic target.

3 Other Lymphomagenic Viruses

3.1 Epstein-Barr Virus

The association of EBV with several specific lymphoid malignancies is quite consistent, indicating an etiopathogenic role in their development. EBV infection is practically ubiquitous in healthy adults, so it has been difficult to establish the exact role of this virus in lymphomagenesis. Nevertheless, extensive epidemiologic and experimental data support the notion that EBV is

an oncogenic virus. EBV can infect and transform normal human B-cells *in vitro*, resulting in their “immortalization” and leading to continuously growing lymphoblastoid cell lines (LCLs) (Rickinson and Kieff 1996). In addition, loss of EBV episomes from transformed lymphoma cell lines results in loss of tumorigenicity.

The pattern of expression of EBV latent genes is associated with specific lymphoma subtypes. In some cases EBV gene expression can be linked specific molecular and oncogenic phenotypes of the lymphoid malignancies. Lymphomas such as Burkitt lymphoma (BL) only express EBNA1. Although EBNA1 transgenic mice have an increased incidence of lymphomas (Wilson et al. 1996), the role of EBNA1 in tumorigenesis is unclear. There is evidence that infection of B cells with EBV lacking EBNA1 can lead to the development of LCLs that are tumorigenic in mice, but this process is much less efficient than with wild-type EBV (Humme et al. 2003). In general it is thought that EBNA1 is not a major oncogene of EBV. BLs bear specific chromosomal translocations involving the *c-myc* oncogene, and these are probably essential because EBNA1 is insufficient to drive cellular proliferation. EBV-associated lymphomas frequently present in immunodeficient individuals, including those infected with HIV and organ transplant recipients. In these, tumor cells usually express a larger number of EBV-encoded genes, which tend to be more oncogenic as well as more immunogenic. These proteins include EBNA2, LMP1, and LM2A, all essential for transformation by EBV *in vitro*. EBNA2 is thought to represent a constitutively active member of the Notch signaling pathway (Grossman et al. 1994; Hsieh and Hayward 1995). The LMP2A protein is structurally reminiscent of two KSHV transmembrane proteins, K1 and K15. Like K1, it contains an ITAM motif, therefore mimicking antigen receptor signaling, and like both, it can signal through specific tyrosine kinases, including *fyn* and *lyn*. The LMP1 protein is transforming and tumorigenic *in vitro* (Wang et al. 1985) and *in vivo*. Transgenic mice expressing LMP1 under the control of immunoglobulin gene regulatory elements develop B-cell lymphomas (Kulwicht et al. 1998). LMP1 functions as a constitutively active CD40 receptor, a member of the TNF (tumor necrosis factor)-receptor family. LMP1 aggregates in the membrane as its cytoplasmic tail interacts with TNF receptor-associated factors (TRAFs) and TNFR-1-associated death domain protein (TRADD), leading to activation of nuclear factor (NF)- κ B and the *c-Jun* amino-terminal kinase (JNK) (Eliopoulos et al. 1999; Kieser et al. 1997; Kilger et al. 1998), a kinase cascade activated by inflammatory cytokines and involved in *bcr-abl* leukemogenesis (Dickens et al. 1997). Thus signaling by EBV LMP-1 is similar to that of KSHV vFLIP; although these two proteins are not structural homologues, they have subverted the very same signaling pathway to promote the survival of infected B cells.

3.2

Human T-Cell Lymphotropic Virus-1 and Adult T-Cell Leukemia/Lymphoma

Human T-cell lymphotropic virus-1, the first described human lymphotropic retrovirus (Poiesz et al. 1980), is the etiologic agent for adult T-cell leukemia/lymphoma (ATLL). This is an often aggressive malignancy of mature CD4+ lymphocytes. Cumulative evidence points to HTLV-1 as the causal agent of ATLL (Cann and Chen 1996; Dalgleish 1998): (1) Seroepidemiologic studies show a close association between ATLL and HTLV-1 infection; (2) throughout malignant progression ATLL cell clonality or oligoclonality correlates with the clonality or oligoclonality of retroviral insertion; (3) HTLV-1 can transform T cells in vitro, resulting in immortalized cell lines; and (4) HTLV-1 can induce cancers in animal models.

Human T-cell lymphotropic virus-1 seems to play a role in the early steps of ATLL malignant progression and not necessarily in the maintenance of the transformed phenotype (Cann and Chen 1996), because ATLL tumor cells lack expression of HTLV-1 genes (Reitz et al. 1983) and carry viral genomes that tend to be heavily deleted (Yoshida et al. 1985, 1984). ATLL tumor cells do have oncogenic alterations in p53 (Cesarman et al. 1992; Pise-Masison et al. 1998) and CDKN2/p16 tumor suppressor genes (Uchida et al. 1998) and chromosomal aberrations (Fukuhara et al. 1983; Maruyama et al. 1990), the complexity of which correlates with tumor aggressiveness (Sanada et al. 1985). Therefore, oncogenesis of ATLL seems to involve an HTLV-1-dependent step in which transformation of T cells results in a polyclonal population of proliferating immortalized T cells, with subsequent acquisition of new oncogenic genetic alterations, clonal expansion, and progression to full malignancy and HTLV-1 independence (Cann and Chen 1996) (Fig. 4). In contrast to other animal retroviruses that transform cells by transduction of oncogenes or activation of oncogenes adjacent to insertion sites, HTLV-1 transforms T cells by a rather complex mechanism that is mediated by its transactivator protein, Tax (Cann and Chen 1996; Felber et al. 1985; Flint and Shenk 1997; Hiscott et al. 1995). Evidence supporting an oncogenic role for Tax stems from its ability to induce T-cell immortalization (Grassmann et al. 1992, 1989) and mesenchymal tumors in transgenic mice (Hinrichs et al. 1987; Nerenberg et al. 1987). Transgenic mice in which the expression of Tax was specifically targeted to T cells developed a large granular T-cell lymphocytic leukemia (Grossman et al. 1995).

Although HTLV-1 Tax is not generally able to directly interact with DNA (Cann and Chen 1996; Lenzmeier et al. 1998), it is able to exert powerful biological responses by increasing the activity of transcription factors that are able to transactivate both the HTLV-1 LTR and genes involved in T-cell proliferation (Felber et al. 1985; Flint and Shenk 1997; Hiscott et al. 1995).

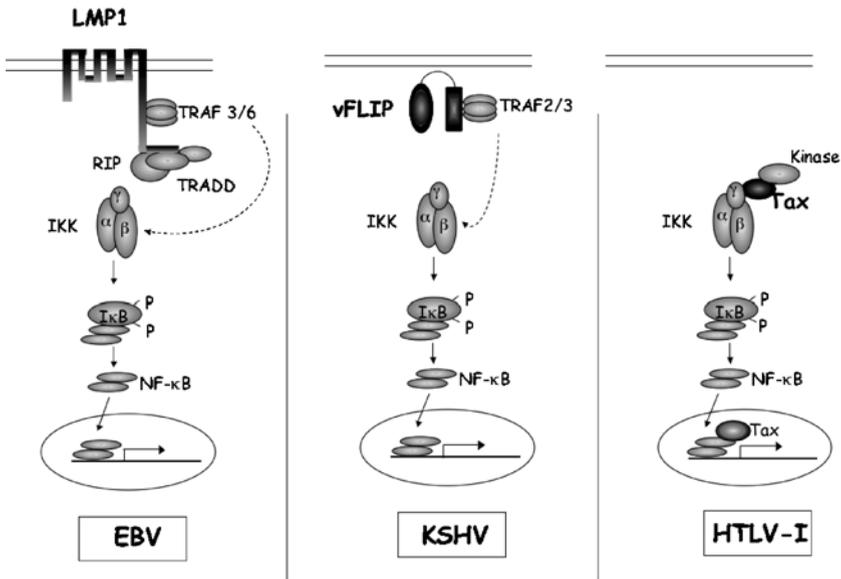


Fig. 4 Viral proteins can activate NF- κ B. Transforming proteins of EBV, KSHV, and HTLV-1 include LMP-1, vFLIP and Tax, respectively. These three proteins share the ability to activate the NF- κ B pathway. Although all three have been shown to activate this pathway through canonical and noncanonical mechanisms, only the canonical pathway is shown. This pathway involves activation of the IKK complex by the corresponding viral protein with subsequent phosphorylation of I κ B α that leads to its degradation. This results in the release of Rel proteins, which are translocated to the nucleus and dimerize to form different NF- κ B complexes that bind to specific sequences to activate the transcription of multiple genes. (Reprinted from Cesarman and Mesri 2006)

Tax can interact with members of the NF- κ B family of transcription factors (Cann and Chen 1996; Flint and Shenk 1997; Leung and Nabel 1988; Ruben et al. 1988), its physiological I κ B inhibitors (Nicot et al. 1998; Petropoulos and Hiscott 1998), and its activating kinases (Geleziunas et al. 1998), leading to increased transcription from NF- κ B-responsive promoters. NF- κ B can activate transcription of many genes related to T-cell proliferation and growth such as *c-rel*, *c-myc*, and both IL-2 and IL-2 receptor. Simultaneous activation of IL-2 and its receptor can lead to IL-2-independent proliferation of T cells expressing Tax (Iwanaga et al. 1999). Although not all HTLV-1-transformed cells express IL-2, the creation of an IL-2 autocrine loop by Tax may be important in the early stages of malignant progression (Cann and Chen 1996).

As in the case of KSHV- and EBV-mediated lymphomagenesis, NF- κ B activation by Tax plays a central role in HTLV-I-mediated oncogenesis. It appears

that HTLV-1 has evolved multiple strategies to subvert the NF- κ B pathway, a major regulator of T-cell survival, activation, differentiation, and proliferation (Yoshida 2001). HTLV-1 Tax can activate NF- κ B through both the canonical or classic and the noncanonical or alternative pathways. HTLV-I Tax can physically interact with IKK γ and activate phosphorylation and degradation of IKK α and IKK β , thus activating NF- κ B through the canonical pathway (Harhaj and Sun 1999; Sun and Ballard 1999). In addition, HTLV-I-transformed cells consistently express p52, indicating that the noncanonical NF- κ B pathway is constitutively activated. Indeed, it was found that HTLV-1 Tax is able to stimulate p100 processing into p52 by bridging the interaction between p100 and IKK α (Xiao et al. 2001). The multiple roles that NF- κ B could play in T-cell tumorigenesis, in addition to the multiple strategies devised by HTLV-I to subvert it, indicate that this pathway is critical for HTLV-1-mediated lymphomagenesis, and therefore it could be an attractive therapeutic target. Bay 11-7082, an NF- κ B inhibitor that acts by inhibiting I κ B α degradation, was shown to induce apoptosis of HTLV-1-infected T-cells but not uninfected T-cells or PMBC (Mori et al. 2002). Moreover, Bay 11-7082 efficiently suppressed ATLL tumor growth in a new model in immunodeficient NOD/SCID gamma-null (NOG) mice (Dewan et al. 2003). These data demonstrated the involvement of the NF- κ B pathway in HTLV-1 tumorigenesis and point to NF- κ B as an attractive target for therapy that can be inhibited to achieve selective cytotoxicity in HTLV-1-associated ATLL tumors

4

Conclusions

KSHV, EBV, and HTLV-1 are associated with specific subsets of malignant lymphomas, and accumulated evidence indicates that they play an etiopathogenic role in their development. Although these viruses differ in many respects, they all carry genes that subvert the host molecular machinery to deregulate cell growth and immortalize infected lymphoid cells, setting the stage for further genetic alterations to take place so that the cells will progress to full malignancy. However, their ability to transform cells and to lead to lymphomagenesis is highly dependent on host factors. Understanding the oncogenic and immunologic mechanisms involved in the pathobiology of virus-associated lymphomas is important for the development of targeted therapeutic and preventive approaches.

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The Use of Antiviral Drugs in the Prevention and Treatment of Kaposi Sarcoma, Multicentric Castleman Disease and Primary Effusion Lymphoma

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1	Introduction	290
2	In Vitro and Animal Model Evidence for the Efficacy of Antiviral Drugs for KSHV	291
2.1	DNA Synthesis Inhibitors	291
2.2	Antiretroviral Therapy	293
2.2.1	Reverse Transcriptase Inhibitors	293
2.2.2	Protease Inhibitors	293
2.3	Summary of Preclinical Work	294
3	Prevention of Kaposi Sarcoma with Antiviral Agents	295
3.1	Herpesvirus Antiviral Medications	295
3.2	Antiretroviral Therapy	297
3.3	Conclusions About KS Prevention	297
4	Treatment of KSHV-Associated Disease with Antivirals	298
4.1	Kaposi Sarcoma	298
4.2	Primary Effusion Lymphoma	300
4.3	Multicentric Castleman Disease	300
5	Conclusions and Future Directions	300
	References	302

Abstract Kaposi sarcoma-associated herpesvirus [KSHV, also known as human herpesvirus 8 (HHV-8)] is the most recently identified member of the human herpesvirus family. Kaposi sarcoma (KS), primary effusion lymphoma, and multicentric Castleman disease are all associated with KSHV infection. Although the incidence of KS has declined dramatically in areas with access to highly active antiretroviral therapy, it remains the most common AIDS-associated malignancy in the developed world and is one of the most common cancers in developing nations. Current treatment options

for KSHV-associated disease are ineffective, unavailable, or toxic to many affected persons. A growing body of basic science, preclinical, and observational data suggests that antiviral medications may play an important role in the prevention and treatment of KSHV-associated disease.

1

Introduction

Kaposi sarcoma (KS) was one of the initial harbingers of the human immunodeficiency virus (HIV) pandemic in 1981 (Center for Disease Control 1981; Hymes et al. 1981). At that time, one in three patients with HIV in North America and western Europe developed KS. Chemotherapy was the mainstay of treatment, leading to clinical resolution in less than half the cases (Krown 2004). By 1994, when Kaposi sarcoma-associated herpesvirus [KSHV, also known as human herpesvirus 8 (HHV-8)] was first identified as the cause of KS (Chang et al. 1994), the incidence had already begun to decline (Eltom et al. 2002). The widespread introduction of highly active antiretroviral therapy (HAART) in the developed world in 1996 was accompanied by a tenfold reduction in the number of new KS cases (Eltom et al. 2002). The precipitous decline in the prevalence of KS shortly after the identification of KSHV, coupled with the challenges of propagating KSHV in culture and the lack of an animal model, has led to few new advances in the treatment of KSHV-related disease. HAART, either alone or in conjunction with conventional chemotherapy, remains the mainstay of treatment for HIV-associated KS. To date, no large randomized trials support the use of one therapy over another for the treatment or prevention of KS, and the efficacy of these treatments is not known. Furthermore, treatment of HIV-negative KS, or other KSHV-associated diseases such as multicentric Castleman disease (MCD) and primary effusion lymphoma (PEL), is based primarily on anecdotes. Worldwide, KS is the most common cancer in areas where coinfection with HIV and KSHV is highly prevalent, and the frequency with which MCD or PEL occurs in the developing world is not known.

Since the introduction of (minimally toxic) antiviral therapy over 25 years ago, numerous clinical studies have documented the efficacy of DNA synthesis inhibitors against human herpesvirus infections. A substantial body of evidence has accumulated supporting a role for antiviral agents in the treatment and prevention of KSHV-associated disease, providing optimism that novel therapies for these diseases may be found.

2 In Vitro and Animal Model Evidence for the Efficacy of Antiviral Drugs for KSHV

2.1 DNA Synthesis Inhibitors

Antiviral therapy was revolutionized in 1978 with the synthesis of acyclovir, a drug that could selectively inhibit viral DNA replication with minimal toxicity to humans (Schaeffer et al. 1978). Acyclovir is a synthetic guanine analog, which after triphosphorylation is incorporated into the 3' end of actively replicating DNA, leading to chain termination. The specificity of acyclovir activity is attributed to the high avidity of acyclovir for many human herpesvirus thymidine kinases, which are capable of efficient phosphorylation, and the relative inefficiency of cellular kinases in performing the initial phosphorylation of the drug. Although all wild-type human herpesviruses are thought to possess the thymidine kinase enzyme, not all accept acyclovir as a substrate, leading to significant differences in the ability of acyclovir to inhibit DNA synthesis. The valine ester derivative of acyclovir, valacyclovir, as well as the chemically similar penciclovir and its oral prodrug, famciclovir, have similar mechanisms of action and substrate specificities. Ganciclovir and its valine ester derivative, valganciclovir, compete with cellular dGTP for incorporation into the growing viral DNA chain. These drugs differ in their avidity for the viral kinase, with increased activity against cytomegalovirus (CMV). Foscarnet prevents the binding of pyrophosphate to the viral DNA polymerase through inhibition of the phosphotransferase enzyme, resulting in termination of replication in a thymidine kinase-independent manner. Finally, cidofovir is a cytosine analog that only requires diphosphorylation to cause chain termination after incorporation by the viral DNA polymerase, independent of the action of thymidine kinase.

The nucleotide sequence of KSHV suggests that the virus encodes both a thymidine kinase (TK) (ORF 21) and a phosphotransferase (PT) (ORF 36). These regions share several domains that are conserved among human herpesviruses; the KSHV TK and PT have sequence homologies of nearly 25% when compared with Epstein Barr virus (EBV), and 12%–16% of the nucleotides are shared with HSV and VZV (Cannon et al. 1999). Additionally, domains within the KSHV TK and PT regions show homology to other human herpesvirus sequences that are known to confer susceptibility to ganciclovir. 293 cells transfected with KSHV ORF 21 or ORF 36 are capable of phosphorylating ganciclovir (Cannon et al. 1999). Based on these early observations, a number of in vitro experiments have been performed to determine whether antiherpetic antivirals inhibit KSHV replication.

Although “classic” *in vitro* antiviral susceptibility testing relies on observing a reduction in viral cytopathic effect (CPE) in cell culture, the inability to observe CPE in KSHV-infected cell lines necessitates novel approaches. The identification of an immortalized cell line that is persistently infected with latent KSHV (BCBL-1), and the recognition that KSHV could be stimulated to lytic replication by the addition of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Renne et al. 1996), has allowed for limited studies of antiviral activity. The amount of KSHV viral DNA produced after stimulating replication with TPA, as quantified by electrophoresis and Southern blotting, was reduced in the presence of ganciclovir (Kedes and Ganem 1997; Medveczky et al. 1997; Neyts and De Clercq 1997), foscarnet (Kedes and Ganem 1997; Medveczky et al. 1997; Neyts and De Clercq 1997), and cidofovir (Kedes and Ganem 1997; Medveczky et al. 1997), with acyclovir having minimal effect (Kedes and Ganem 1997; Medveczky et al. 1997; Neyts and De Clercq 1997). The estimated concentrations at which 50% of viral DNA was inhibited (IC_{50}) ranged from 60 to 80 μM for acyclovir [peak serum concentration 100 μM after administration of 10 mg/kg intravenously (IV)], from 2 to 5 μM for ganciclovir (peak serum concentration 50 μM after administration of 5 mg/kg IV or 900 mg oral valganciclovir), from 0.05 to 1 μM for cidofovir (peak serum concentration 30–70 μM after administration of 5 mg / kg IV), and from 80 to 500 μM for foscarnet (peak serum concentration approximately 1,000–6,000 μM after administration of 90 mg/kg IV). Similar results were described after examining the amount of viral DNA produced with real-time polymerase chain reaction (Friedrichs et al. 2004). Estimates of inhibitory concentrations using flow cytometry to identify cells expressing the lytic envelope protein K8.1 differed significantly, however, with cidofovir (EC_{50} of 3.9 μM) and foscarnet (EC_{50} 74.7 μM) being identified as the only active agents. Other drugs had estimated inhibitory concentrations that would not be sufficiently exceeded with typical administration (acyclovir 38.3 μM and ganciclovir 53.7 μM).

It is not known whether the degree to which antiviral drugs inhibit lytic reactivation from latency in an immortalized cell line mimics antiviral activity *in vivo*. However, two other systems have been established to examine antiviral activity against KSHV. Human microvascular endothelial cells are infected with KSHV in patients with KS (Dupin et al. 1999) and may be important in the initiation of tumorigenesis (Carroll et al. 2004). In a primary cell line of human microvascular endothelial cells, foscarnet completely suppressed the production of infectious KSHV after primary infection with KSHV (Krug et al. 2004). In another approach, immunocompromised mice implanted with human fetal thymus and liver grafts (SCID-hu Thy/Liv) (McCune et al. 1988) allow productive infection with KSHV after direct inoculation with virus harvested from BCBL-1 cells (Dittmer et al. 1999). The intraperitoneal ad-

ministration of ganciclovir both prevented the establishment of infection and also decreased the amount of latent and lytic KSHV mRNA detected after infection was established.

2.2

Antiretroviral Therapy

The incidence of KS in the United States began to decline in 1987 (Eltom et al. 2002), shortly after the approval of zidovudine for the treatment of HIV. More dramatic reductions were seen after 1995, when the use of protease inhibitors (PI) became common (Eltom et al. 2002). The mechanism of this effect has been thought to be multifactorial, including the deaths of many persons infected with HIV in the early epidemic, the immune reconstitution seen with antiretroviral therapy, or perhaps a direct effect of the drugs against KSHV. Both direct and indirect effects of antiretroviral therapy on KSHV have been studied *in vitro* and are summarized below.

2.2.1

Reverse Transcriptase Inhibitors

Two experiments have cloned the KSHV TK and used bacterial expression vectors to assess the ability of this enzyme to phosphorylate HIV nucleoside reverse transcriptase inhibitors (NRTI). The thymidine analogs zidovudine (Gustafson et al. 2000; Lock et al. 2002) and stavudine (Lock et al. 2002) both were phosphorylated by KSHV TK and competitively inhibited the incorporation of radiolabeled thymidine.

2.2.2

Protease Inhibitors

HIV PIs prohibit the assembly of HIV virions by occupying the active site of an aspartyl protease. Herpesviruses use a serine protease during lytic replication to begin assembly of viral particles, which would not be affected by HIV PIs (Shimba et al. 2004). Coculturing BCBL-1 cells with indinavir had no effect on the expression of latent or lytic KHSV genes before or after stimulation with TPA, supporting that the effect of these drugs on KS was not through a direct effect on KSHV replication (Sgadari et al. 2003).

It is plausible that several mechanisms may mediate both direct and indirect effects of PIs on KSHV replication. For example, the use of PIs is associated with a reduction in the amount of HIV-1 Tat, which has been shown to increase KSHV replication (Huang et al. 2001). Ritonavir suppressed the ability

of HIV-1 Tat to induce transcriptional activation of nuclear factor κ B (NF- κ B), which is thought to lead to the development of KS (Pati et al. 2002).

In addition to affecting KSHV replication, PIs may prevent the development of KS from KSHV-infected cells. Coculturing KSHV-infected immortalized cells with ritonavir enhanced their apoptosis (Pati et al. 2002). The use of the SCID-hu mouse model confirmed that ritonavir (Pati et al. 2002), saquinavir (Sgadari et al. 2002), and indinavir (Sgadari et al. 2002) inhibited KS tumor formation after infection with KSHV. Both saquinavir and ritonavir prevented the invasion of human vascular endothelial cells through the basement membrane, an important initial step in angiogenesis and KS tumorigenesis.

Finally, the immune reconstitution seen with HAART may have profound effects in controlling KSHV infection. Cytotoxic lymphocyte (CTL) responses have been examined for seven KSHV proteins, with an increase in CTL to two KSHV proteins in subjects receiving HAART (Wilkinson et al. 2002). Lysis of KSHV latently infected cells by NK cells is deficient in AIDS patients with progressive KS but is restored after HAART (Sirianni et al. 2002). Taken together, these studies support an indirect effect of ART on KSHV through improved cellular immunity.

2.3

Summary of Preclinical Work

Taken together, the body of *in vitro* and animal model work supports the ability of certain antiviral agents to suppress KSHV replication and prevent establishment of productive infection. Furthermore, the HIV PIs may block some of the early steps that are thought to be essential to initiating tumorigenesis. The translatability of these findings to treating or preventing disease in humans may be tempered by a number of factors.

First, agents that suppress DNA replication would not be expected to have an effect on early KSHV gene products, which are produced by a protein in the KSHV virion and not by viral DNA polymerase, or latent KSHV genes that are transcribed by human DNA polymerase. Many of these gene products may play an important role in the development of human disease, such as the immediate-early gene products viral interleukin 6 (vIL-6) or the viral G protein-coupled receptor (vGPCR), and the latency-associated nuclear antigen (ORF 73). Gene array technology confirms that cidofovir has little effect on early gene products, including vIL-6 and vGPCR, but effectively reduces production of a number of late structural genes (Lu et al. 2004). Additional experiments show that acyclovir, foscarnet, and cidofovir all reduce the ability of BCBL-1 cells induced with TPA to express the late gene product of K8.1, a virion glycoprotein (Zoetewij et al. 1999). These drugs did not alter the

expression of the early gene product ORF 59, a nonstructural protein that is thought to bind DNA polymerase to assist in transcription. Similarly, it has recently been described that in BCBL-1 cells valproic acid induces both lytic replication of KSHV and apoptosis (Klass et al. 2005). Coculturing the cells with foscarnet or ganciclovir inhibited the production of the late structural gene ORF26 but had no effect on vIL-6 or vGPCR. As discussed below, the clinical relevance of these findings is unclear. Antiviral medications have been shown to be associated with improvements in symptoms from MCD, which directly follow reductions in KSHV viral load (Casper et al. 2004)

Second, the role that KSHV replication plays in clinical disease is not clear. In the cases of KS or PEL, it could be argued that KSHV latent and lytic gene products are essential for malignant transformation, but once transformation has occurred their role is minimized. Examination of KS tissue shows that only 5% of cells harbor lytic virus; whether this activity is required for tumor maintenance is not known.

A small number of studies have evaluated the utility of antiviral therapy in the prevention and treatment of KSHV-related disease, as discussed below.

3 Prevention of Kaposi Sarcoma with Antiviral Agents

Additional indirect evidence for antiviral efficacy against KSHV derives from cohort studies conducted early in the HIV epidemic (Table 1). Patients treated with either antiretroviral agents or DNA synthesis inhibitors early in the HIV epidemic were witnessed to have significant reductions in the risk of developing KS.

3.1 Herpesvirus Antiviral Medications

Among 3,688 HIV-positive men in England, the risk of developing KS in persons using intravenous foscarnet or ganciclovir for the treatment of CMV retinitis was reduced by over 60% (Mocroft et al. 1996). Similarly, among 935 HIV-positive American men enrolled in the Multicenter AIDS Cohort Study, the risk of developing KS was reduced by 44% with ganciclovir use and 60% with foscarnet (Glesby et al. 1996). Neither study found acyclovir use to be associated with a lower risk of developing KS. A separate study of high-dose acyclovir for the treatment of HIV showed no reduction in the incidence of KS among persons treated with acyclovir (Ioannidis et al. 1998). A comparison of oral to intravenous ganciclovir administered for the treatment of HIV-associated CMV retinitis found that the risk of developing KS was reduced

Table 1 Clinical studies evaluating the efficacy of antiviral medications in the prevention of Kaposi sarcoma

Study	Antiviral medication(s)	Results
Mocroft (1996)	Foscarnet Ganciclovir Acyclovir	Among 3,688 HIV-positive men in England: <ul style="list-style-type: none"> • 72% reduction in the chance that persons receiving ganciclovir or foscarnet would develop KS compared with those not receiving either drug (HR 0.38 and 0.39, respectively). • Acyclovir not found to be associated with reduced incidence of KS (HR 1.1).
Glesby (1996)	Foscarnet Ganciclovir Acyclovir	Among 935 HIV-positive men in the US, incidence of KS reduced by: <ul style="list-style-type: none"> • 60% with foscarnet • 44% with ganciclovir Acyclovir was not found to be associated with reductions in KS.
Ionnidis (1998)	Acyclovir	Meta-analysis of 8 trials including 1,792 patients found no reduction in the odds of developing KS among persons taking acyclovir.
Martin (1999)	Oral vs. intravenous ganciclovir	Risk of developing KS was reduced 75% among HIV-positive men receiving oral ganciclovir for CMV retinitis and 93% in those receiving intravenous ganciclovir.
Joffe (1997)	Zidovudine	Meta-analysis of trials evaluating high-dose zidovudine for the treatment of HIV found 36% reduction in the rate of KS development.
Joffe (1997)	Zidovudine	Analysis of 1,847 men from Multicenter AIDS Cohort Study found no increase in time to KS among persons receiving zidovudine.
Portsmouth (2003)	HAART containing PI vs. NNRTI	Prospective study of 1,204 HIV-positive persons found risk of developing KS was reduced 58% among those receiving NNRTIs and 53% with PIs (difference not statistically significant).
Stebbing (2004)	Ritonavir-containing HAART	No significant difference in the incidence of KS among English HIV-positive men treated with and without ritonavir-containing HAART

KS, Kaposi sarcoma; HR, hazard ratio; CMV, cytomegalovirus; HAART, highly active antiretroviral therapy; PI, protease inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor

75% and 93%, respectively, compared with an intravitreal ganciclovir implant alone (Martin et al. 1999). Although these data are encouraging, no randomized trial of antivirals for KS prevention has been reported, and the efficacy in preventing KS in persons without advanced HIV, or preventing MCD or PEL, remains unknown.

3.2

Antiretroviral Therapy

Several randomized, placebo-controlled trials of high-dose zidovudine (≥ 1000 mg daily) for the treatment of HIV demonstrated a 36% reduction in risk of developing KS compared with persons receiving placebo alone (reviewed in Joffe et al. 1997). Participants in these trials were extremely heterogeneous with respect to their stage of HIV and indication for zidovudine. An analysis of incident KS in the prospective Multicenter AIDS Cohort Study found no reduction in KS associated with zidovudine use, after adjustment for the indication for initiating ART and changes in CD4 count (Joffe et al. 1997).

Subsequent analyses have examined the effect of combination antiretroviral therapy on the incidence of KS. The large Swiss HIV Cohort Study found that the standardized incidence ratio (SIR) for KS among HIV-positive persons was reduced nearly 10-fold for those who used HAART before developing KS (SIR for HAART users 25.3 vs. 23.9 for nonusers) (Clifford et al. 2005). The risk of developing KS was reduced 26% among 1,204 HIV-positive English men who used NRTIs alone, 53% in those on HAART regimens with nonnucleoside reverse transcriptase inhibitors (NNRTI), and 58% among men taking PIs after adjustment for age, ethnicity, and nadir CD4 count before developing KS (Portsmouth et al. 2003). In contrast to what might be expected from *in vitro* data, there was no statistically significant difference in the effect of NNRTIs vs. PIs in preventing KS, and regimens containing ritonavir were no more effective in KS prevention (Stebbing et al. 2004). These findings suggest that immune reconstitution may be an important component of the improvements in KS that have been well documented after initiation of HAART.

3.3

Conclusions About KS Prevention

The consistent and compelling data from international cancer registries documenting reductions in the incidence of KS with the introduction of antiviral therapy, coupled with supportive basic science research, argue that KS can be prevented in those at highest risk for its development. To date, apart from immunosuppression, the only risk factor for the development of KS has been the detection of KSHV in the peripheral blood (Whitby et al. 1995; Campbell

et al. 2000; Broccolo et al. 2002; Gill et al. 2002; Lorenzen et al. 2002; Cannon et al. 2003). As discussed below, the treatment of KSHV-infected patients with either PIs (Leao et al. 2000) or ganciclovir (Casper et al. 2004) is associated with reductions in KSHV viral load. Prospective clinical trials are needed to determine whether antiviral therapy reduces the incidence of epidemic, classic, or endemic KS in KSHV-viremic persons.

4

Treatment of KSHV-Associated Disease with Antivirals

4.1

Kaposi Sarcoma

Shortly after their introduction, case reports indicated that the use of PIs was associated with remission of AIDS-associated KS (Conant et al. 1997; Murphy et al. 1997; Diz Dios et al. 1998; Jung et al. 1998; Krischer et al. 1998; Lebbe et al. 1998; Martinelli et al. 1998; Tavio et al. 1998). Subsequent larger observational series evaluated regimens with both PIs and NNRTIs (Table 2). Among 78 patients with AIDS-associated KS, HAART with either a PI or a NNRTI prolonged the time to receipt of additional topical or systemic therapy, compared with treatment courses in these same patients before initiating HAART (Bower et al. 1999). Twelve of 14 (86%) of patients previously treated with ART or chemotherapy had remissions in their KS documented at a median of 4 months after initiating PI-based ART; of note, the two patients who failed to respond experienced similar increases in CD4 count but had persistent HIV viremia (Cattelan et al. 2001). These observations were corroborated by a separate study of 26 KS patients, of whom 22 (85%) achieved at least a partial response (Pellet et al. 2001). KSHV viremia was also significantly reduced among persons on PIs, which was independently predictive of KS improvement (Leao et al. 2000; Pellet et al. 2001). Finally, two studies documenting the relapse (Bani-Sadr et al. 2003) and new onset (Rey et al. 2001) of KS after switching from PI-based to NNRTI-based HAART suggest that the efficacy of all ART in treating KS may not be the same. To date, no randomized prospective trials have evaluated the frequency and predictors of KS regression among persons treated with HAART (Krown 2004).

The efficacy of DNA synthesis inhibitors in treating KS is less well established. The largest trial to date used intravenous cidofovir to treat seven patients with advanced KS (T_1 disease or >50 lesions), including five HIV-positive patients (4 on HAART), and two patients with classic KS (Little et al. 2003). No patient had either a complete or a partial response to cidofovir, and the median time to progression was 8.1 weeks.

Table 2 Clinical studies evaluating the efficacy of antiviral medications in the treatment of Kaposi sarcoma

Study	Treatment	Findings
Bower (1999)	HAART with PI or NNRT vs. no HAART	<ul style="list-style-type: none"> ● 78 patients with HIV-associated KS had the time from first to second topical or systemic treatment of KS evaluated before and after starting HAART. ● Before HAART, median time to second treatment was approximately 9 months, which increased significantly to 1.7 years after initiation of HAART. ● No significant difference in time to second treatment among persons on NNRTIs vs. PIs ● Persistent HIV viremia while on HAART was the strongest predictor of KS development.
Cattelan (2001)	HAART with PI	<ul style="list-style-type: none"> ● 86% of 14 patients with HIV-associated KS had response to HAART with PIs. ● Median time to response was 6 months. ● Response predicted by absence of HIV viremia
Pellet (2001)	HAART with PI	<ul style="list-style-type: none"> ● 85% of 26 patients with HIV-associated KS had response to HAART with PIs. ● Median time to response was 251 days ● Response predicted by lower KSHV viral load and increases in CD4 counts
Badi-Sadr (2003)	HAART	<ul style="list-style-type: none"> ● 5 patients with HIV-associated KS had regression of KS documented with initiation of PI-containing HAART. ● All 5 patients relapsed at a median of 11 months when switched to NNRTI-containing HAART, despite little change in CD4 count. ● Relapse after NNRTI was not associated with HIV virologic failure.
Rey (2001)	HAART with PI vs. efavirenz	<ul style="list-style-type: none"> ● One of 62 HIV-positive patients developed KS after switching from PI to efavirenz.
Little (2003)	Cidofovir	<ul style="list-style-type: none"> ● None of 5 patients with HIV-associated KS or 2 patients with classic KS responded to cidofovir. ● Median time to progression was 8.1 weeks

KS, Kaposi sarcoma; HAART, highly active antiretroviral therapy; PI, protease inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor

4.2

Primary Effusion Lymphoma

The relative rarity of PEL makes definitive evaluations of its treatment challenging, and evidence for the efficacy of antiviral therapy comes from case studies alone (Table 3). The first report described a 38-year-old man with HIV-associated PEL, admitted with hemophagocytic syndrome, who was successfully treated with ganciclovir and chemotherapy (Pastore et al. 2000). Subsequently, additional reports described the successful treatment of PEL with intravenous cidofovir and interferon α (Hocqueloux et al. 2001) or intrapleural cidofovir in three elderly Italian HIV-negative patients (Luppi et al. 2005).

4.3

Multicentric Castleman Disease

Of all the KSHV-associated diseases, MCD may be associated with the highest degree of active lytic replication (Katano et al. 2000, 2001). In support of this, clinical flares of MCD are consistently accompanied by KSHV viremia, which resolve with effective therapy (Grandadam et al. 1997; Bottieau et al. 2000; Oksenhendler et al. 2000; Corbellino et al. 2001; Boivin et al. 2002; Berezne et al. 2004; Casper et al. 2004). Our group initially showed that treatment of three patients with KSHV/HIV-associated MCD with ganciclovir or valganciclovir led to rapid resolution of clinical symptoms and viremia (Casper et al. 2004). Two of the three experienced disease-free intervals of over 1 year. In contrast, a subsequent series showed that five patients with HIV-associated MCD failed to respond to the combination of cidofovir and chemotherapy (Berezne et al. 2004). The role of antiretroviral therapy in the treatment of MCD is also poorly defined. One series of seven cases showed no relationship between ART, CD4 count, and the course of MCD (Aaron et al. 2002). A second, however, suggested that the initiation of ART in three patients with MCD could lead to a fulminant and fatal course (Zietz et al. 1999) (Table 3).

5

Conclusions and Future Directions

In the decade since the first description of KSHV, the combination of basic science research, retrospective epidemiologic studies, and clinical observations supports a role for antiviral therapy in the prevention and treatment of KSHV-related disease. Similar to what has been described with Epstein-Barr-associated malignancy, antiviral therapy is likely to have the greatest impact on the prevention of oncogenesis. In areas of the world where KS remains

Table 3 Treatment of primary effusion lymphoma and multicentric Castleman disease with antiviral medications

Study	Disease	Therapy	Findings
Pastore (2000)	PEL	Chemotherapy and ganciclovir	<ul style="list-style-type: none"> ● Report of HIV-positive patient with hemophagocytic syndrome and PEL who responded to intravenous and oral ganciclovir, HAART, cyclophosphamide, vincristine, and prednisone
Hocquelooux (2001)	PEL	Cidofovir, interferon- α , HAART	<ul style="list-style-type: none"> ● Report of HIV-positive patient with PEL who responded to combination therapy including intravenous cidofovir
Luppi (2005)	PEL	Cidofovir	<ul style="list-style-type: none"> ● Series of 3 patients with classic KS received 2–3 doses of intrapleural cidofovir for thoracic PEL. ● All 3 had remission, as documented by repeat radiologic studies of the chest.
Casper (2004)	MCD	Ganciclovir or valganciclovir	<ul style="list-style-type: none"> ● 3 patients with HIV-associated MCD experienced clinical and virologic responses to treatment with intravenous ganciclovir or oral valganciclovir.
Berezne (2004)	MCD	Cidofovir and chemotherapy	<ul style="list-style-type: none"> ● 5 patients with HIV-associated MCD experienced recurrent symptoms despite cidofovir and either vinblastine or etoposide.
Aaron (2002)	MCD	HAART with PI	<ul style="list-style-type: none"> ● 7 patients with MCD had recurrent flares despite increases in CD4 counts and HIV virologic suppression in 6/7. ● 4/7 had comorbid KS, which resolved in 3/4. ● 1 patient failed to respond to chemotherapy, ganciclovir, cidofovir, foscarnet, splenectomy, or corticosteroids.
Zietz (1999)	MCD	HAART	<ul style="list-style-type: none"> ● 3 patients developed symptoms of MCD and died shortly after initiating unspecified HAART regimen.

PEL, primary effusion lymphoma; MCD, multicentric Castleman disease; HAART, highly active antiretroviral therapy; PI, protease inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor

a prevalent disease, future studies should be directed at the identification of persons at highest risk for developing KS from asymptomatic KSHV infection and determining the optimal antiviral prevention strategies. Although far less common, antiviral therapy may be most promising in the prevention and treatment of the more “active” KSHV diseases, PEL or MCD. Finally, it is clear that even within a class of antiviral drugs, there may be significant heterogeneity in the clinical response to their use for a given disease. As such, comparative trials of antiviral or antiretroviral therapy would be of great benefit to those afflicted with KSHV-associated disease.

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Interactions Between HIV-1 Tat and KSHV

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1	Introduction	309
2	HIV-1 Tat	310
3	KSHV Infection	313
4	HIV-1 Versus HIV-2	314
5	Interactions Between Tat-1 and KSHV	315
6	Conclusions	317
	References	318

Abstract Since the advent of the HIV-1 pandemic, a close association between HIV-1 infection and the development of selected types of cancers has been brought to light. The discovery of Kaposi sarcoma-associated herpesvirus (KSHV) has led to significant advances in uncovering the virological and molecular mechanisms involved in the pathogenesis of AIDS-related malignancies. Extensive evidence indicates that HIV-1 *trans*-activating protein Tat plays an oncogenic role in the development of KSHV-associated neoplasms. Comprehensive knowledge of the functions of Tat-1 together with the KSHV genes will contribute to a better understanding of the pathogenesis of virus-associated cancers and the interaction of viruses with their hosts.

1 Introduction

HIV-1 infection predisposes to the development of specific types of cancer. Most neoplasms that arise during states of immunodeficiency are associated with oncogenic virus infections, such as Epstein-Barr virus (EBV), human papillomavirus (HPV) and Kaposi sarcoma (KS)-associated herpesvirus (KSHV). For this reason, a diminished immune surveillance against viruses and virus-infected tumor cells is believed to contribute to the development of these malignancies.

The introduction of highly active antiretroviral therapy (HAART) has dramatically changed the landscape of HIV-1 disease. HAART brings about a substantial and sustained decrease in peripheral blood HIV-1 load, as well as an increase in CD4⁺ T cells and a significant decrease in the incidence of AIDS-defining KS (Mocroft et al. 2000; Nasti et al. 2003; Palella et al. 1998). Patients with advanced KS treated with chemotherapy concurrently with HAART have achieved long-term survival (Holkova et al. 2001; Mocroft et al. 2000; Nasti et al. 2003; Palella et al. 1998), and some KS lesions regress after initiation of HAART (Aoki et al. 2004a; Krown 2004).

Importantly, however, the onset of AIDS-KS is not always associated with reduced peripheral blood CD4⁺ T cell counts. In addition, KS is more aggressive in AIDS than in other immunodeficiency states, including solid organ transplant recipients, implying that factors other than increased KSHV replication due to immunodeficiency are involved. Also, HPV-associated cervical cancer develops in HIV-infected patients irrespective of host immune status. However, disease progression is enhanced by HIV, suggesting that HIV-1 acts as a cofactor promoting cancer growth (Clarke et al. 2002). Experimental evidence has shown that the *trans*-activating protein Tat from HIV-1 has unusual properties for a transcriptional factor in that it can up-regulate expression of a number of viral and cellular genes, act as a proangiogenic factor, enhance KSHV transmission to target cells, and cooperate with KSHV-derived proteins in promoting tumorigenesis (Albini et al. 1996b; Ambrosino et al. 1997; Aoki et al. 2004a; Badou et al. 2000; Bennasser et al. 2002; Scala et al. 1994; Watson et al. 1999). This review discusses pathogenetic mechanisms underlying KSHV-associated neoplasms, taking into account direct and indirect effects of HIV-1 Tat on tumorigenesis.

2 HIV-1 Tat

HIV-1 encodes three structural proteins (matrix, capsid, and nucleocapsid), two envelope proteins (gp120 and gp41), three enzymes (protease, reverse transcriptase, and integrase), two regulatory proteins (Tat and Rev), and four accessory proteins (Nef, Vpr, Vpu, and Vif) (Frankel et al. 1998). After two decades of extensive investigation, remarkable progress has been achieved in the understanding of the molecular mechanisms mediating the various activities of HIV-1-derived proteins, particularly in characterizing the Tat protein in relation to cancer development (Watson et al. 1999).

The HIV-1 Tat protein, the *trans*-activating factor of HIV-1, is a polypeptide comprising 86–101 amino acids (aa), which is encoded by 2 exons (Kup-

puswamy et al. 1989; Watson et al. 1999). The first 72 amino acids, encoded by the first exon, possess the full *trans*-activating activity of Tat (Garcia et al. 1988). Structure-function studies have distinguished the first 72 residues of Tat into five major domains (Fig. 1). The cysteine-rich region mediates the formation of metal-linked dimers in vitro and is essential for Tat function (Frankel et al. 1988; Garcia et al. 1988; Ruben et al. 1989). The basic region is important for the nuclear localization of Tat and contributes to Tat *trans*-activating function (Calnan et al. 1991; Hauber et al. 1989; Rubartelli et al. 1998). Within the basic region, which comprises residues 49–57, a nuclear localization signal, GRKKR, has been identified (Ruben et al. 1989). As a result, Tat accumulation in the nucleus is rapid, occurring in a few minutes (Watson et al. 1999). The carboxy-terminal amino acids encoded by the second exon contain an RGD motif, which is important for Tat binding to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins (Barillari et al. 1993). It was proposed that through this RGD-mediated integrin engagement, the carboxy-terminal region is indispensable for Tat function as a proangiogenic factor in vitro and in vivo (Mitola et al. 2000).

Tat is a potent transactivator of HIV-1 gene expression and is essential for viral replication. Biochemical studies have demonstrated that Tat binds to the HIV-1 promoter located immediately downstream of the transcriptional start-site at the 5' end of the nascent viral RNA transcript at the *trans*-activation responsive (TAR) element (Delling et al. 1991; Roy et al. 1990). Despite its lacking a secretory signal Tat is released from HIV-1 infected cells, and extracellular Tat has the ability to enter the uninfected cell and transactivate endogenous genes, such as tumor necrosis factor, interleukin (IL)-2, and IL-6 (Ambrosino et al. 1997; Chen et al. 1997; Ehret et al. 2001; Rubartelli et al.

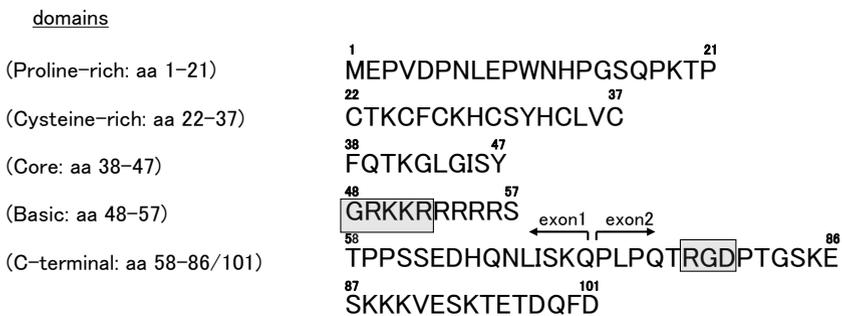


Fig. 1 Domain structure of HIV-1 Tat. The complete amino acid sequence of the Tat polypeptide is shown, and the five major domains are indicated. The first 72 aa are encoded by exon 1 and the rest are encoded by exon 2. The nuclear localization signal and the RGD motif are shaded

1998; Westendorp et al. 1994). The addition of hydrophobic groups to Tat was shown to increase the protein uptake by cells (Chen et al. 1995). Through this and perhaps other ill-defined mechanisms, Tat has been reported to *trans*-activate other cellular genes (Frankel et al. 1998; Rubartelli et al. 1998) and to regulate collagen expression and cell survival-related proteins including p53 and Bcl-2 (Li et al. 1995; Zauli et al. 1995). Moreover, Tat can carry biologically active proteins into cells in a receptor- and transporter-independent fashion and can elicit different biological responses in distinct target cells (Rubartelli et al. 1998; Schwarze et al. 2000; Vives et al. 2003). This shuttle function is mediated principally by Tat's basic domain.

Tat has a net positive charge and, owing to its strong electrostatic interaction, binds to negatively charged molecules, including cell-associated heparan sulfate proteoglycans, immunoglobulin-like domains of vascular endothelial growth factor (VEGF) receptor (VEGFR)-2, and chemokine receptors (Albini et al. 1996b; Rusnati et al. 2000; Tao et al. 1993). Binding of Tat to VEGFR-2 promotes angiogenesis *in vivo*, which is strongly potentiated by heparin (Albini et al. 1994). Many proangiogenic factors are heparin-binding proteins, including fibroblast growth factors and VEGF, and their activity is dependent on the presence of heparin or heparan sulfate (Albini et al. 1996a, 1996b; Klagsbrun et al. 1991; Tessler et al. 1994; Yayon et al. 1991). As is the case with fibroblast growth factor (Sommer et al. 1989), the tight binding to heparin may protect Tat from proteolytic degradation and disclose the proangiogenic activity of Tat at concentrations at which Tat alone is not effective (Albini et al. 1996a).

There is evidence supporting a role for Tat as a cofactor in the pathogenesis of AIDS-associated KS. *In vitro*, Tat is a growth factor for KS spindle cells, and transgenic overexpression of Tat in mice promotes formation of KS-like lesions (Ensoli et al. 1990; Huang et al. 1993). When human KS cell lines are injected into mice, tumor cells grow faster in the presence of circulating HIV-1 Tat (Prakash et al. 1997). However, this effect may not be restricted to KS as Tat could indirectly promote tumor cell growth by promoting tumor angiogenesis. Tat also appears to affect cell adherence and anchorage-dependent cell growth through its RGD integrin-binding motif. Close contact of AIDS-KS with normal vascular cells is facilitated by Tat via a specific interaction of the Tat RGD sequence with integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, a function that is augmented by Tat basic domain (Barillari et al. 1993).

In addition to displaying proangiogenic activity in several systems, Tat was reported to induce apoptosis of primary human brain microvascular endothelial cells, which resulted in increased endothelial cell permeability (Kim et al. 2003). The mechanism by which Tat induces apoptosis in selected endothelial cells is not presently known.

3 KSHV Infection

The discovery of KSHV within KS lesions from HIV-seropositive and -seronegative individuals (Moore et al. 1995) and a number of subsequent studies have provided strong evidence for an essential role of KSHV in the pathogenesis of KS (Boshoff et al. 2002). KSHV is also believed to play a role in the pathogenesis of primary effusion lymphoma (PEL) and a proportion of cases of multicentric Castleman disease (MCD) (Aoki et al. 2000a, 2004a, 2000c, 2001c). Histologically, KS lesions consist of a proliferation of spindle cells mixed with inflammatory cells, and are characterized by the formation of hypervascular structures and enhanced vascular permeability (Antman et al. 2000). In KS lesions, KSHV is present in the vast majority (>90%) of spindle cells and in the surrounding lymphatic or neoangiogenic vessels (Dupin et al. 1999; Katano et al. 2000, 1999). However, the virus is not detectable in well-formed established vascular endothelium or in neighboring normal dermis (Dupin et al. 1999). This has been attributed to the fact that KSHV is mostly latent in KS tissues, and in part to the difficulty at transmitting KSHV infection to normal noninfected cells.

This limited transmission rate of KSHV is supported by *in vitro* observations in various assay systems. Endothelial cell infection with KSHV *in vitro* has been explored in detail because most studies concur that KS cells are of endothelial lineage, as inferred by their expression of endothelium-specific molecules (Karp et al. 1996). In general, there are two avenues for viral transmission: direct contact between target and virus-producing cells (cell-mediated transmission) and cell-free transmission by viral particles (Dimitrov 2004; Spear et al. 2003). Most groups using cell-free KSHV transmission system have prepared highly concentrated KSHV particles harvested from phorbol acetate-induced PEL cell lines, mixed viral preparations with adhesive agents such as Polybrene, and added the mixture onto the endothelial cells (Akula et al. 2002; Flore et al. 1998; Moses et al. 1999; Poole et al. 2002; Tomescu et al. 2003; Vieira et al. 2001). Transmission efficiencies have varied significantly among cell types, and long-term infection has often been difficult (Grundhoff et al. 2004). Cell-mediated KSHV transmission was successfully demonstrated by direct coculture of KSHV-infected PEL cells with primary human vascular endothelial cells (HUVECs) (Sakurada et al. 2001). With this system, KSHV was detected in HUVECs up to 30 days after the removal of PEL cells. In this model, however, 10-fold more PEL cells than HUVECs were required for successful virus transmission (Sakurada et al. 2001), suggesting that the virus may not infect endothelial cells efficiently. When cells from KS lesions are placed into culture, KSHV is lost in a few passages while out-

growth of virus-negative spindle cells is quite rapid. As a consequence, all known KS-derived cell lines are virus negative.

Although impaired immune surveillance may result in increased KSHV replication in infected patients, there is a significantly higher incidence of KS in AIDS than in other immunodeficiency states. Compared to the general population, immunosuppressed patients after solid organ transplantation display an increase up to 500-fold in the incidence of KS, whereas male homosexual AIDS patients display an increase of KS incidence up to 20,000-fold. It is thus possible that HIV-1-related factors promote KSHV transmission. We have found that Tat can accelerate KSHV transmission into endothelial cells (Aoki et al. 2004a). The essential fragment mediating this effect is the 13-amino acid basic region of Tat (Aoki et al. 2004a). In vitro experiments indicate that Tat can increase KSHV transmission into cells at protein concentrations 10-fold higher than those reported present in sera from HIV-1-infected individuals (Aoki et al. 2004a). However, Tat concentrations in lymph nodes of HIV-1-infected individuals are expected to be substantially higher than those found in sera from HIV-1-positive individuals because HIV-1 load in lymphoid tissues exceeds by orders of magnitude that found in the bloodstream (Cavert et al. 1997; Hockett et al. 1999; Pantaleo et al. 1998, 1993). The occurrence of most intense HIV-1 replication in lymphoid tissues and the association of primary KSHV infection with the development of lymph nodal KS in an HIV-1-infected patient (Oksenhendler et al. 1998) suggest that lymphoid tissues are a likely preferential site of KSHV spread. This hypothesis that KS precursor cells or KSHV-infected cells originate at a distant site and reach the site of KS development, most commonly the skin, is supported by recent studies in transplant recipients (Barozzi et al. 2003) and in the experimental setting (Yao et al. 2003).

4

HIV-1 Versus HIV-2

Although the incidence of various malignancies is clearly increased in HIV-1-infected individuals, there are no definitive cancer statistics for HIV-2-infected individuals (Bock et al. 2001). In contrast to HIV-1 infection, HIV-2 infection is associated with a longer asymptomatic period, lower plasma viral loads, and lower rates of heterosexual and perinatal transmission than those of HIV-1 (Kanki et al. 1994; Marlink et al. 1994; Popper et al. 1999; Schim van der Loeff et al. 1999). HIV-2 infection is not commonly associated with KS development, even when accompanied by KSHV infection (Ariyoshi et al. 1998; Weiss et al. 2000). The incidence of KS is higher in African countries where HIV-1 is prevalent than in countries where HIV-2 is more common, despite similar

seroprevalence of KSHV. HIV-1 is pandemic, but HIV-2 is more endemic, with stable prevalence rates in most countries. HIV-2 transmission appears to differ from that of HIV-1. Heterosexual transmission is responsible for the majority of HIV-2 infections worldwide, although a few reports of homosexual HIV-2 transmission have been documented (Cilla et al. 2001; Schim van der Loeff et al. 1999). In addition, HIV-2 transmission is estimated to occur at a much lower rate than that of HIV-1: Sexual and vertical forms of HIV-2 transmission are about five- to ninefold and 10- to 20-fold reduced relative to HIV-1, respectively (Reeves et al. 2002). Thus the difference between routes of HIV-1 and HIV-2 viral transmission may be related, in part, to differences in KS incidence between HIV-1- versus HIV-2-infected individuals (Ariyoshi et al. 1998).

Another potential explanation for the epidemiological findings is that Tat proteins encoded by HIV-1 and HIV-2 (Tat-1 and Tat-2, respectively) differ in their ability to serve as cofactors in KSHV-induced KS development. Tat-2 is a polypeptide consisting of 130 residues encoded by 2 exons. Like Tat-1, a basic region of Tat-2 encoded by exon 1 binds to a TAR-RNA-binding domain at the 5' end of viral transcripts and stimulates viral transcription (Chang et al. 1992). However, exon 2 of HIV-2 Tat increases the binding affinity of Tat-2 to HIV-2 TAR RNA and contributes to *trans*-activation of HIV-2 LTR (Chang et al. 1992; Pagtakhan et al. 1995; Rhim et al. 1994). Exon 2 of Tat-2 encodes distinct domains, including the amino-terminal, cysteine-rich, and core regions (Pagtakhan et al. 1995; Rhim et al. 1994). Although the latter two regions have greater than 65% amino acid sequence homology with the corresponding regions of Tat-1, the amino-terminal region of Tat-2 has only 10% homology to Tat-1 (Pagtakhan et al. 1997). In addition, Tat-2 lacks an RGD motif, which is important for Tat-1 integrin binding (Bock et al. 2001). Although Tat-1 and Tat-2 show considerable similarity, Tat-2 is unable to effectively transactivate the HIV-1 promoter (Berkhout et al. 1990). The Tat-1 protein, however, can effectively transactivate the wild-type HIV-2 promoter (Rhim et al. 1995). Tat-1 is not a stronger transactivator than Tat-2, as fusion protein studies suggest that Tat-2 is the more potent transactivator if it can be adequately recruited to the promoter (Rhim et al. 1993). Detailed analysis of structural and functional differences between Tat-1 and Tat-2 may help understand the mechanisms by which HIV-1 acts as a potent cofactor in the development of certain cancers.

5 Interactions Between Tat-1 and KSHV

The critical role of KSHV infection in KS pathogenesis is well established, but many aspects of this process are incompletely understood. KSHV encodes

numerous open reading frames with striking homology to cell regulatory genes (Choi et al. 2001). However, KSHV gene expression in KS lesions is highly restricted, and the pattern of viral mRNA in most KS tumor cells is consistent with latent infection (Katano et al. 2000).

Viral FLICE-inhibitory protein (vFLIP: ORF K13/71) is one of the few viral proteins to be expressed in latently infected KS spindle cells and PEL cell lines (Godfrey et al. 2005; Guasparri et al. 2004; Katano et al. 2000). Cells expressing vFLIP are protected against apoptosis induced by Fas or tumor necrosis factor receptor-1 (Djerbi et al. 1999). A recent study showed that vFLIP is largely responsible for constitutive nuclear factor (NF)- κ B activation in PEL cells (Guasparri et al. 2004). As in other virus-associated lymphomas, constitutive activation of both the NF- κ B and signal transducer and activator of transcription-3 appears to be essential for PEL cell survival (Aoki et al. 2003a; Keller et al. 2000). In addition to vFLIP, viral G protein-coupled receptor (vGPCR: ORF74) and ORF K1 can activate NF- κ B. However, these viral transcripts are predominantly expressed during the lytic phase (Cannon et al. 2003; Cesarman et al. 2000; Samaniego et al. 2001). Tat-1 is a potent NF- κ B activator (Demarchi et al. 1996), which can bind directly to NF- κ B enhancer sequence (Dandekar et al. 2004) and may thus contribute to PEL cell survival in HIV-1-infected individuals.

Although most HIV-1-infected cells are not co-infected with KSHV, experimental results suggest that selected KSHV-encoded proteins may influence the life cycle of HIV-1. KSHV Rta (ORF50) was shown to increase HIV-1 infectivity (Varthakavi et al. 2002). Latency-associated nuclear antigen (LANA: ORF73) was reported to activate HIV-1 long terminal repeat (Hyun et al. 2001). In an HIV-1-infected patient with MCD, circulating levels of viral IL-6 (vIL-6: ORF K2) correlated directly with circulating levels of HIV-1 RNA (Aoki et al. 2001b). In addition, it is possible that HIV-1-derived proteins can activate KSHV lytic replication, although the role of Tat-1 alone is controversial (Inoue et al. 2003; Varthakavi et al. 2002).

KSHV and Tat-1 may also cooperate to modify cellular gene expression. One important target of exogenous Tat-1 is the vascular or lymphatic endothelium. KSHV infection of endothelial cells promotes the expression of VEGF-A and VEGF-C as well as their receptors VEGFR-2 and VEGFR-3 (Hong et al. 2004; Masood et al. 2002; Wang et al. 2004). Tat-1 also activates VEGFR-2 and VEGFR-3, and by this mechanism, can induce angiogenesis and stimulate KS spindle cells (Albini et al. 1996b). In addition, Tat-1 induces the expression of adhesion molecules and matrix metalloproteinases, which play a critical role in KS progression (Aoki et al. 2003c; Lafrenie et al. 1997). vIL-6, which is not expressed in most KS cells but is elevated in sera from HIV-1-positive patients with KS (Aoki et al. 2000a, 2001c), directly activates KS

cells (Klouche et al. 2002) and stimulates VEGF-A production *in vitro* and *in vivo* (Aoki et al. 1999a). VEGF-A is an essential growth factor for PEL cell survival *in vivo* (Aoki et al. 1999b, 2001a, 2000b). Tat-1 has been shown to induce the expression of IL-6 and IL-10, which serve as autocrine growth factors for MCD and PEL, respectively (Beck et al. 1994; Jones et al. 1999). Transgenic vGPCR mice develop KS-like tumors (Guo et al. 2003). A cell line derived from one such tumor expresses vGPCR and forms tumors in nude mice (Yang et al. 2000). Transfection of the *tat-1* gene into these tumor cells increases NF- κ B and NF-AT activation levels, and accelerates tumor formation (Guo et al. 2004).

In certain hematopoietic cells, Tat-1 can upregulate the expression of the chemokine receptor CXCR4 (Gibellini et al. 2003). Its ligand, stromal-derived factor-1 (SDF-1), which is constitutively expressed by skin capillary endothelium, can trigger specific arrest of KSHV-infected cells under physiological shear flow conditions (Yao et al. 2003). By triggering specific adhesion of circulating KSHV-infected cells and favoring their entry into the extravascular cutaneous space, endothelial cell-associated SDF-1 in cutaneous capillaries may contribute to the occurrence of KS in the skin.

Taken together, these results suggest that Tat-1 can contribute to the high incidence and aggressive nature of KS in AIDS, presumably by promoting KSHV infection. The impact of HAART on the occurrence and severity of KS may be due, at least in part, to suppression of Tat-1 protein expression.

6 Conclusions

Based on the advances in our understanding of HIV-1-related disorders, HIV-1 Tat emerges as a very important protein for its role not only in HIV-1 infection but also in cancer development. Understanding the direct and indirect roles of Tat-1 in the pathogenesis of KSHV-infected neoplasms could provide means to help us design novel strategies for prevention and treatment of virus-induced pathology. Tat-1 targeting may represent yet another avenue for future clinical application in certain KSHV-related disorders.

A role for HIV-1 in the pathogenesis of KS is supported by the observation that the introduction of HAART has dramatically decreased the incidence of KS. In contrast, KSHV-positive MCD in AIDS often progresses to fatal disease in spite of HAART (Aoki et al. 2001b, 2001c; Dupin et al. 2000; Zietz et al. 1999). Furthermore, the impact of HAART on AIDS-associated EBV-related lymphomas and HPV-related cervical cancer is variable (Clarke et al. 2002; Kirk et al. 2001; Levine et al. 2000), suggesting that additional factors

are involved in the pathogenesis of these neoplasms. Further advances in identifying these additional factors may lead us to uncover novel facets of HIV-1 infection and its role in the initiation and progression of malignant disorders.

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Subject Index

- adhesion molecules 140
- Amerindian 14, 35
- angiogenesis 139, 142
- animal model 218
- anti-CD20 255
- antiviral gene 222
- apoptosis 196–199

- B cells 218
- Bantu expansion 12, 36
- BCBL cells 221

- c-Kit 226
- Castleman disease 138
- CBP 190, 191, 194
- CD31 228
- cell cycle 139, 145
- cidofovir 255
- contamination 250
- cytomegalovirus (CMV) 147

- discovery 213

- EBV 146
- EC infection
 - bone marrow-derived 219
 - DMVEC 219
 - HUVEC 219
 - primary 219
 - spindle morphology 220
- endothelial cell 211, 218
 - E6/E7-DMVEC 223, 230
 - life-extended EC 222
 - primary EC 222
 - TIME cells 227, 230
- epidemiological form 212

- episomal maintenance 218
 - insufficiency 218
- episome maintenance 224
- explant 218, 227

- febrile illnesses
 - children 12
- foscarnet 255

- G protein 138, 139, 142
- ganciclovir 255
- Ganda tribe 21
- gene expression 220, 221, 229
 - host 220, 221
 - profiling 225
 - viral 220
- Gleevec 226
- GPCR-specific kinases (GRK) 142

- HCMV genome 3
 - multisite analysis 3
- heme oxygenase-1 226
- herpesvirus evolution
 - chimeric 6
- histone acetyltransferase (HAT) 191, 192, 194
- HIV-1 Tat 141
- Homo neanderthalis* 32
- host shut-off 227
- human herpesvirus-6 and -7 (HHV-6, -7) 147
- human papillomavirus 223
- Hwalian 8, 14, 25
- hypervariable
 - VIP and TMP loci 17
- hypervariable domain 4

- ICAM-1 228
 immune evasion 228
 immunofluorescence assays 252
 – latent 252
 – lytic 252
 immunosuppression 253
 in vitro 211
- K12 214, 215
 K5 *see* MIR2
 Kaposin *see* K12
 Khoisan 34–36
 KS lesion 212, 231
 – model 231, 233
 KSHV 250
 KSHV infection 213
 – BEC 230
 – bone marrow-derived 219
 – DMVEC 219–222
 – E6/E7-DMVEC 223–226, 230
 – experimental 218
 – HUVEC 219
 – in vivo 215
 – LEC 230
 – low MOI 222
 – morphologic change 224
 – primary 219
 – TIME cells 227, 228, 230
- LANA-1 223
 – p53 214, 223
 – Rb 214
 LANA-1 *see* ORF73
 LANA-2 225
 LANA-2 *see* ORFK10.5
 latency 214
 latent infection 215, 222, 224, 225
 lymphatic EC 219, 228, 229
 – reprogramming 221
 lymphocryptovirus 37
 lymphocyte 211
 lytic gene 216
 lytic infection 224
 lytic reactivation 225, 231
 – ORF 50 215
 – phorbol esters 215
 – sodium butyrate 215
- lytic replication 143–145, 215, 216
- MHC-1 228
 microarray 220, 221, 225, 226, 230
 migration 5
 – out of Africa 22, 36
 MIR2 221
 mitogen- and stress-activated protein
 kinases 142
 multicentric Castleman
 disease 213
- Neanderthal 6, 27, 38
 – modern humans 6, 33, 34
 – out of Africa 33, 34
 Neuritin 226
 nonsynonymous rates 4
- ORF45 199
 ORF50 215
 ORF71 214
 ORF72 214
 ORF73 214
 ORFK10.5 214, 221
- p300 190, 191, 194
 Pacific Rim 5, 13, 14, 16, 19, 20, 24,
 25, 31, 33, 34
 – modern humans 38
 pathogenesis 229, 230
 PDGF-R 226
 PEL 216
 PEL line 217, 219, 220
 phylogenetic tree
 – VIP protein 8
 PKR 189, 193, 198, 199
 PML bodies 192
 Polynesian 14, 25, 35
 posttransplant KS 246
 – heart 248
 – incidence of 247
 – kidney transplantation 247
 – liver 248
 primary effusion lymphoma
 138, 213
 Prox-1 228

- RDC-1 226
- reactivation 248
- recombination 27
 - junction 27, 32
- recombination event 36
 - chimeric 16
 - cross-species 6, 38
 - junction 16
- roseolovirus 37
- Rta *see* ORF0215

- screening 254
- seroconversion 250
- serological assays 252
- seroprevalence 213
- spindle cells 140, 212, 224, 229
 - origin 229
 - precursors 219

- sub-Saharan Africa 2, 5, 6, 10, 12, 13, 19, 24, 25, 35, 36
 - mitochondrial 16
 - modern humans 16
 - out of Africa 16

- therapeutic strategy 217, 226, 231
- transformation 225
- tumorigenesis 216, 231

- vascular EC 219, 229
- vCyclin *see* ORF72
- vFLIP *see* ORF71
- vGPCR paracrine effects 139, 140, 143, 144

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