REGULATION OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV) LYTIC GENE EXPRESSION BY VIRAL TRANSACTIVATOR RTA AND CELLULAR REPRESSOR K-RBP

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Kaposi's sarcoma-associated herpesvirus (KSHV) is a newly identified human herpesvirus and the etiologic agent of Kaposi's sarcoma (KS). KSHV can establish latency and be reactivated to lytic replication in infected cells. The viral replication and transcriptional activator (RTA) is the key protein that initiates KSHV lytic replication by activating the expression of many KSHV lytic genes and DNA replication. Understanding how RTA transactivates target genes and is modulated by cellular factors is pivotal to elucidate the mechanism by which KSHV regulates its latent and lytic replication cycles.

KSHV-RTA binding protein (K-RBP) is a cellular RTA interacting protein. It contains a Kruppel-associated box (KRAB) at the N terminus and 12 adjacent zinc finger motifs. K-RBP is a transcriptional repressor and down-regulate RTA-mediated transactivation of several KSHV promoters including ORF57. The KRAB domain is the repression domain and the zinc finger domain binds DNA with high GC content. Moreover, K-RBP binds to and suppresses KSHV ORF57 promoter in a GC-rich element-dependent manner. K-RBP also competes with RTA in binding to ORF57 promoter. In addition, the zinc finger domain of K-RBP is sufficient to suppress RTA-

mediated transactivation of ORF57 promoter suggesting an important role of the DNA-binding activity of K-RBP in repressing RTA-mediated transactivation. K-RBP is also a negative regulator of RTA-mediated KSHV lytic replication.

Further studies show that RTA promotes K-RBP degradation through a proteasome-dependent pathway. RTA also stimulates degradation of several other repressors. The ability of RTA to induce degradation correlates with its ability to transactivate target promoter. These results suggest that KSHV RTA stimulates the turnover of repressors to favor viral lytic gene expression and lytic replication.

Together these studies suggest that the cellular transcriptional repressor K-RBP suppresses RTA-mediated transactivation and KSHV reactivation, while RTA promotes the degradation of the repressor to stimulate KSHV lytic replication. Finally, these studies suggest that the establishment of latency or lytic replication involves a regulatory loop between RTA and the repressors.

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List of Abbreviations

aa: amino acid

AD: activation domain

AHV-1: alcelaphine herpesvirus 1

AIDS: acquired immune deficiency syndrome

Bcl-2: B-cell leukemia/lymphoma 2

BD: binding domain

BHV-4: bovine herpes virus 4

BSA: bovine serum albumin

C/EBPα: CCAAT/enhancer binding protein alpha

CBP: CREB-binding protein

ChIP: Chromatin Immunoprecipitation

CHX: cycloheximide

HCMV: human cytomegalovirus,

cpm: counts per minutes

CREB: cAMP response element-binding

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DTT: dithiothreitol

EBSS: Earle’s Balanced Salt Solution

EBV: Epstein-Barr virus,

EDTA; ethylenediamine tetraacetic acid

EHV-2: equine herpesvirus type II

EMSA: electrophoretic mobility shift assay

FBS: fetal bovine serum

GADD45α: growth arrest DNA-damage-inducible alpha

GFP: green fluorescent protein

h: hour

HAT: histone acetyltransferase

HDAC1: histone deacetylase

HHV8: human herpesvirus 8,

HIF1α: hypoxia inducible factor alpha

HIV-1: human immunodeficiency virus type 1,

hKFC: human counterpart of kinase from chicken C

HMGB1: high mobility group box 1

HVS: herpes virus saimiri

ICP0: infected cell protein 0

IE: immediate early

IFN: interferon

IRF-7: IFN regulatory factor 7

ISG: interferon stimulated gene

KAP-1: KRAB-Associated Protein 1

kb: kilobase

kDa: kilodalton

KRAB: Krüppel-associated box

K-RBP: KSHV-RTA binding protein

Krim: KRAB box protein interacting with Myc

KRIP-1; KRAB-A interacting protein 1

KS: Kaposi's sarcoma,

KSHV: Kaposi's sarcoma-associated herpesvirus

LANA: latent nuclear antigen

LC3: microtubule-associated protein1 light chain 3

MCD: multicentric Castleman's disease,

γHV-68: murine gammaherpesvirus 68

min: minute

MOI: Multiplicity Of Infection

MxA: myxovirus resistance protein A

NFIB: nuclear factor I/B

NF-κB: nuclear factor-kappaB

NLS: nuclear localization signal

Oct-1: Octamer 1

ORF: open reading frame

PAN: polyadenylated nuclear

PARP-1: poly (ADP-ribose) polymerase 1

PBS: phosphate buffered saline)

PCR: polymerase chain reaction,

PEL: primary effusion lymphoma,

PKA: protein kinase A

PMSF: phenylmethylsulphonylfluo

Rb: retinoblastoma

RBP-Jκ: recombination signal-binding protein 1 for Jkappa

RFP: red fluorescent protein

Rit1: ras-like protein 1

RNA: ribonucleic acid

RRE：RTA responsive element

RRV: rhesus rhadinovirus

RTA: regulation and transcription activator

SAAB: selected and amplified binding sites

SD: synthetic dropout medium

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SETDB1: SET domain bifurcated 1

STAF50: stimulated trans-acting factor of 50 kDa

Stat3: signal transducer and activator of transcription 3

SWI/SNF: switch/sucrose nonfermentable

TBST: Tris-buffered saline Tween-20

TIF1β: transcriptional intermediary factor 1 beta

TK: thymidine kinase

TPA: 12-O-tetradecanoyl-phorbol-13-acetate

TRAP: thyroid hormone receptor (TR)-associated protein

TSA: trichostatin A

Ub: ubiquitin

vFLIP: viral (FLICE)/caspase-8-inhibitory protein

vGPCR: viral G protein-coupled receptors

VHL: von Hippel Lindau

vIL-6: viral Interleukin 6

vMIP-I: viral macrophage inflammatory protein type 1

XBP-1: X-box binding protein 1

YY1: Ying Yang 1

ZBRK1: zinc finger and BRCA1-interacting protein with a KRAB domain 1

ZFP: zinc finger protein

Chapter 1: Introduction and Literature Review

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), is a newly identified human herpesvirus and the etiologic agent of Kaposi's sarcoma (KS). KS is the most common neoplasm in AIDS patients (26, 44). Similar to other herpesviruses, KSHV can establish latency and be reactivated from latency in infected cells (30). The viral lytic replication plays an important role in the development of KS (129, 133). The lytic reactivation of KSHV is tightly controlled by the expression of a viral gene open reading frame 50 (ORF50), which encodes the immediate early protein known as replication and transcription activator (RTA). RTA is sufficient and necessary to initiate KSHV lytic replication by activating the expression of a number of lytic genes (87, 132). The activation of target genes by RTA involves the direct DNA binding of RTA (85, 127). In addition, numerous cellular and viral factors were found to modulate RTA-mediated transactivation through various mechanisms (146). However, the mechanism of RTA transactivation of the target gene expression and how RTA is modulated by cellular factors is only incompletely understood.

This dissertation centers on the interaction between KSHV RTA and a novel cellular KSHV-RTA binding protein (K-RBP) (141), and the biological consequences of the interaction in regulating KSHV lytic replication and latency. It contains literature review and results. The literature review is divided into six sections. The first three sections briefly introduce the KSHV biology and related diseases. The following three sections describe the gene expression and regulation during the KSHV life cycle and focus on how RTA expression is regulated, the mechanisms of RTA-mediated

transactivation and how RTA function is regulated by cellular and viral factors. The results are divided into five chapters from chapter two to chapter six. In chapter two, the novel cellular protein K-RBP was characterized as a transcriptional repressor and a negative regulator of RTA-mediated transactivation and KSHV lytic replication. In chapter three, the mechanism of K-RBP repression of RTA-mediated transactivation was further studied. K-RBP was found to be a DNA-binding protein and bind KSHV ORF57 promoter. RTA binding to this promoter can be competed by the K-RBP protein. The binding and competition by K-RBP contribute to repression of the KSHV ORF57 promoter. In chapter four, RTA was found to be able to overcome K-RBP-mediated suppression by promoting K-RBP degradation through the ubiquitin-proteasome pathway. This mechanism was also extended to other repressors since RTA induced the degradation of some other repressors. In chapter five, the yeast two-hybrid screening was performed to identify K-RBP interacting proteins to further study K-RBP function. One of the proteins, beclin 1, which is an autophagic protein, was further studied. Finally, in chapter six, we concluded that the cellular transcriptional repressor K-RBP suppresses RTA-mediated transactivation and KSHV reactivation, while RTA promotes the degradation of the repressor to stimulate KSHV lytic replication based on the results. The potential future directions are also described.

Literature Review

-Regulation of KSHV lytic gene expression and replication

1. KSHV-associated diseases.

KSHV has been found to associate with three neoplastic disorders: Kaposi's Sarcoma (KS), Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD).

1.1. Kaposi's Sarcoma (KS).

KS was first documented as an angiomatous neoplasm in elderly men in the Mediterranean region by Moritz Kaposi in 1872, which was later classified as classic or sporadic KS (57). Today, there are three other known forms of KS discovered: endemic KS in the sub-Saharan region, epidemic (or AIDS-associated) KS and iatrogenic KS (in organ and tissue transplant recipients receiving immunosuppressive therapy) (57). It is well established that KSHV is the etiologic agent for all forms of KS (1, 4, 121) after the finding that KSHV was associated with the KS of a patient with AIDS by Chang and her colleagues in 1994 (26). KSHV DNA can be detected in 95% of KS cases (45, 88). However, KSHV is likely a necessary but insufficient factor for KS development, since not all infected individuals develop KS and the development of KS is more likely in certain populations, such as immunosuppressed patients (138). The mechanism by which KSHV causes diseases and its cofactors is not completely understood.

Classic KS is a rare disease occurring in elderly men. The incidence of classic KS in the Mediterranean region is 10-fold higher than in other regions of Europe (38, 50, 58). The incidence is particularly high in Italy, Turkey, Greece and Israel (58). However, even within the same country, the distribution of KS in different geographic regions varies. For example, the islands of Sardinia and Sicily have much higher KSHV infection rates and KS incidents as compared to other regions of Italy (117). These specific geographical

distributions of KSHV and KS suggest that there are risk factors which facilitate the infection, transmission and disease progression in these particular areas.

Epidemic KS has increased dramatically since the AIDS epidemic in the early 1980's (91). It has been the most common neoplasm in AIDS-associated malignancies (44). Even though the new KSHV infection and KS incidence have declined in recent years in the United States due to the successful use of highly active antiretroviral therapy (HAART), KS remains a major problem in AIDS patients in sub-Saharan Africa (123). The increased incidence of KS in AIDS patients suggests that human immunodeficiency virus-1 (HIV-1) infection is a cofactor for KSHV to cause disease. However, it is unknown whether KSHV takes advantage of immune suppression, or plays a role in the destruction of the immune system in AIDS patients.

Endemic KS was found in the African continent prior to the emergence of the HIV epidemic, and mainly affected young men and children (139). The AIDS pandemic in the 1980s dramatically increased the incidence of KS. For instance, in the pre-AIDS Uganda of 1950s and 1960s, KS accounted for about 6.5% of male cancers, while it accounted for 48.6% of all male cancers in 1989 and 1991 (139).

The iatrogenic KS or transplant associated KS occurs in patients infected with KSHV after transplants and were treated with immunosuppressive drugs. This form of KS can either go through a chronic or rapid disease course after the initial KSHV infection (57).

1.2. Primary Effusion Lymphoma (PEL).

KSHV also associates with PEL, a rare monoclonal B cell lymphoma (19). Eighty percent of PEL is also co-infected with another herpesvirus, Epstein-Barr virus (EBV) (69) . Several cell lines, such as BC-1, BC-3 and BCBL-1, established from PEL contributed significantly to the study of KSHV because they can stably maintain the KSHV genome and can be reactivated from latency to produce viral particles for the molecular and serologic studies on KSHV.

1.3. Multicentric Castleman's Disease (MCD).

In addition to KS and PEL, KSHV was found in 50% of Multicentric Castleman's disease (MCD), a rare B cell lymphoproliferative disorder (128). Interestingly, almost 100% of MCD incidence in AIDS patients is KSHV positive (48).

2. Taxonomy of KSHV.

KSHV is the eighth and most recently discovered herpesvirus. The genomic sequence of KSHV was completed and it was classified as a member of gamma-herpesviruses, known as lymphotropic viruses (26, 112). There are two genera in gamma-herpesviruses: gamma-1 (lymphocrytovirus) and gamma-2 (rhadinovirus) herpesviruses. KSHV belongs to the gamma-2 herpesvirus genus and the closest human herpesvirus relative of KSHV is EBV, which is the prototype of gamma-1 herpesvirus. The other known gamma-2 herpesviruses include: herpes virus saimiri (HVS), a primate herpes virus; murine herpesesvirus-68 (MHV-68); bovine herpesvirus-4 (BHV-4); equine herpesvirus-2 (EHV-2); alcelaphine hepesvirus-1 (AHV-1); rhesus rhadinovirus (RRV) (146). Since there is no good animal model to study KSHV, the other gamma-

herpesviruses, such as RRV and MHV-68, have been used to study the pathogenesis in their respective natural hosts (98, 109).

3. The natural history and transmission of KSHV.

Primary infections by KSHV are usually asymptomatic (40). In the primary KSHV infection studies in children with febrile illness, KSHV DNA are only detected in a few children, which suggests a potential KSHV primary infection (63). Following primary infection, immunoglobulin (Ig) M and IgG antibodies against KSHV antigens are detected and the levels of antibody titer may be a predictor of KSHV-associated diseases (91). Primary infections in individuals lead to different outcomes. In most people with normal immune function, KSHV infection does not cause significant disease and the KSHV DNA copy number is low. However, in patients who are immunosuppressed, KSHV has a much greater opportunity to cause disease (46). The incubation periods for disease development caused by KSHV infection vary from months to years in those immunosuppressive patients (99). The viral loads and DNA copy numbers are much higher in KS lesions as compared to normal tissues (100).

At present, the routes of KSHV transmission have yet to be fully understood. Sexual transmission is suggested to be the major transmission route in North American and some northern European countries (91). However, nonsexual transmission routes are also suggested by several studies. Blood transfusion has been linked to KS development in a study in San Francisco (91). Studies have also demonstrated that transplantation is a route for KSHV transmission (104, 110). The detection of KSHV DNA in saliva in a

study using Zambian patients suggests a transmission route through saliva (7). More studies should be done to further elucidate the route of KSHV transmission.

4. KSHV genomic organization and gene expression.

KSHV contains a large double-stranded DNA genome approximately 165kb in size. This large DNA genome contains a long 140.5 kb unique region flanked by 20-35kb terminal repeat regions consisting of 801 bp high GC (84.5%) content repeated subunits (112). There are nearly 90 open reading frames (ORFs) which have been identified in the KSHV genome and all of them are encoded in the long unique region (40). Those ORFs that are the homologs of HVS were assigned the same number as in HVS, while those ORFs that are unique to KSHV were assigned numbers with the prefix K.

Like other herpesviruses, KSHV can establish latent infection in infected cells and be reactivated to lytic replication (30). The gene expression pattern of KSHV is also similar to other herpesviruses. KSHV genes are usually classified into two categories: latent genes and lytic genes (111, 118, 160). During latency, the KSHV genome exists as a closed circular episome with only a limited number of genes expressed, including latency-associated nuclear antigen (LANA), v-cyclin, vFLIP, Kaposin and LANA2 (vIRF2) (10, 39, 67, 96, 108, 113, 119). Recent studies also demonstrated that there are a number of KSHV-encoded microRNAs expressed during latency (14, 15, 90, 105, 106, 115, 116). These proteins and microRNAs expressed during latency were suggested to function in viral episome persistence, viral genome replication, viral immune evasion and viral latency (31, 42, 70, 72, 81, 82, 115, 122, 124, 136). Upon reactivation, the viral genome replicates via a rolling circle mechanism and the genome is cleaved at the

terminal repeat region (112). The lytic genes can be further divided into three subcategories based on the expression kinetics during lytic replication: immediate early (IE), early and late genes. The expression of IE genes upon induction or after primary infection does not require *de novo* protein synthesis and occurs in the presence of protein synthesis inhibitor cycloheximide (153, 161). The proteins encoded by IE genes usually function as regulatory factors in viral and cellular gene expression. Several genes have been identified as IE genes in KSHV using chemical inducers and cycloheximide. These genes include ORF50, ORF45, ORFK4.2 and a 4.5kb mRNA (47, 55, 86, 118, 133, 161). The most extensively studied IE gene in KSHV is ORF50. The expression of ORF50 is sufficient and necessary to induce the lytic replication program of KSHV. Some of the KSHV early genes and late genes have also been identified and they are under the control of IE genes (119, 133, 161).

Most of the studies on KSHV gene expression were carried out in B cell lines derived from PEL. There were also studies examining the gene expression in KS, PEL and MCD tumor cells associated with KSHV infection using viral protein-specific antibodies or probes to detect viral transcripts and their encoded gene products. These studies showed that the expression patterns of KSHV in different tumors are much more complicated: different diseases correlate to different viral gene expression programs (29, 62, 64, 65, 102, 103, 129, 131). These results suggest that different gene expression patterns contribute to the development of different tumor types.

The latent gene products of KSHV regulate cell growth, transformation and tumorigenesis in disease progression. The LANA protein binds to the tumor suppressor proteins p53 and pRb, which stimulates cell transformation and blocks apoptosis (13, 42,

66, 122, 137). The other latent proteins can contribute to tumorigenesis by manipulating cell cycle, inhibiting apoptosis or activating NF-κB (3, 43, 49, 51, 93). The lytic gene products of KSHV can also contribute significantly to tumor development. KSHV is undergoing lytic replication in a small population of the KS cells. These lytic gene products include viral macrophage inflammatory protein-I (vMIP-I), viral interlukin 6 (vIL-6) and viral Bcl-2 (129, 133). Thus, lytic replication can facilitate virus spread and create a favorable microenvironment for the growth of tumor cells through the production of viral and cellular cytokines induced during lytic replication. Understanding the molecular events during the switch from latency to lytic replication will help to understand viral pathogenesis and tumor development.

5. Lytic reactivation of KSHV.

The switch from KSHV latency to lytic replication in KSHV can be initiated by a single KSHV IE protein: RTA, which is encoded by the ORF50 gene (87, 132). This is different from EBV, with which the reactivation from latency is induced by the expression of two IE genes: BZLF1 (ZTA) and BRLF1 (RTA) (2). A number of studies show that RTA is the key for KSHV transition from latency to lytic replication. First, ORF50 is one of the IE genes induced upon 12-O-tetradecanoylphorbol-13-acetate (TPA) induction and the ORF50 mRNA can be detected at 1 h post TPA treatment (118, 133). The expression of ORF50 mRNA is also resistant to protein synthesis inhibitor cycloheximide (153). Second, ectopic expression of RTA alone in KSHV latently infected PEL cells is sufficient to disrupt latency and drive KSHV into lytic replication cycle. Many of the KSHV lytic genes can be induced upon expression of RTA, including

KbZIP, ORF57, vIL-6, PAN RNA, ORF59 and K8.1 (47, 87, 97, 132). Third, interference of RTA function reduces the level of viral lytic replication. The dominant-negative mutant RTA which lacks C-terminal activation domain could reduce the spontaneous reactivation of KSHV (86). Moreover, knock-down of RTA expression using the specific ribozyme decreased the reactivation rate in KSHV infected PEL cells (162). Finally, using a KSHV bacterial artificial chromosome (BAC) system, the RTA-deficient KSHV mutant with the ORF50 locus disrupted does not enter lytic replication unless an ectopic RTA is expressed (150). These findings suggest that RTA is both sufficient and necessary to induce KSHV reactivation from latency. The mechanism by which RTA induces KSHV reactivation is due to its ability to initiate the lytic cascade by transactivating the expression of a number of KSHV genes (146). Recently, RTA was found to be incorporated into KSHV virions (6, 75), suggesting that it may function as transactivator immediately after primary infections. In addition, RTA expression stimulates KSHV DNA replication evidenced by the amplification of the KSHV lytic origin in a plasmid-based replication assay (5, 144). The findings suggest that RTA has a direct role in lytic DNA replication. RTA also plays an indirect role in activating the viral genes required for viral DNA replication, such as KbZIP (5, 144). How RTA expression is regulated and how RTA activates target gene expression are the central questions for understanding the switch of KSHV from latency to lytic replication.

5.1. The RTA protein.

KSHV RTA is the homologue of EBV RTA (149). The sequence homology between KSHV RTA and EBV is relatively low. The KSHV RTA protein is 691 amino