Viral Modulators of KSHV Lytic Replication

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ABSTRACT

CARLOS M. GONZÁLEZ: Viral Modulators of KSHV Lytic Replication

(Under the direction of Blossom Damania)

Kaposi's Sarcoma and the B-cell derived primary effusion lymphoma and multicentric Castleman's Disease are all outcomes of infection with Kaposi's Sarcoma–associated Herpesvirus (KSHV). A predominantly latent virus, KSHV also requires a subpopulation of cells to undergo lytic reactivation in order to generate an environment conducive to neoplastic growth. The replication and transcription activator (RTA, Orf50) is necessary and sufficient to initiate the lytic cascade. We have studied two modulators of lytic replication encoded by KSHV Orf49 and Orf64 that support RTA during lytic reactivation.

KSHV Orf49 lies adjacent to and in the opposite strand as Orf50. Upon ectopic expression, Orf49 was found to encode a 30 kDa protein that localized to the cytoplasm and nucleus. The Orf49 transcript displayed early lytic kinetics and was shown to enhance RTA-mediated transactivation of lytic promoters including vGPCR, K8 and Orf57. Orf49 induced c-Jun, JNK and p38 kinase activation. Pharmacologic inhibition demonstrated that activation of the c-Jun and p38 signaling pathways are required for the completion of the lytic cycle, underscoring the importance of KSHV Orf49 during lytic replication.

KSHV Orf64 encodes a large, 2636 amino acid tegument protein. We have found that Orf64 encodes deubiquitinase (DUB) activity in its amino-terminal 200 amino acids *in vitro* and *in vivo*. Whereas all other herpesviral DUBs cleave Lysine 48 (K48)-linked ubiquitin, KSHV Orf64 was capable of cleaving both K48 and K63-linked ubiquitin chains. Cleavage of K63-linked ubiquitin is associated with functional regulation whereas K48-ubiquitination leads to proteosomal degradation. Mutation of a cysteine residue at amino acid position 29 in the catalytic core of the DUB ablated DUB activity. Orf64 also synergized with RTA to augment transcription from RTA-responsive promoters. These data suggest that the deubiquitination function of Orf64 may play an important role in viral replication.

The role of NFκB pathway activation on KSHV pathogenesis in unclear. Various members of this pathway are regulated by ubiquitination and represent potential targets of Orf64. Reactivating KSHV-293 cells selectively activated the canonical NFκB pathway. We discovered that Orf64 induced degradation of IκBα and IKKγ suggesting that it can induce the canonical pathway, highlighting the multiple roles and functions of Orf64.

DEDICATION

To my wife Vanessa, thank you so much for the unwavering encouragement, love and support that made this journey possible. To Wilma, Ethan and Michael, you guys are my joy and inspiration. And to my parents, thank you for everything.

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LIST OF ABREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AMC	7-amido-4-methylcoumarin
bp	Base pairs of DNA
BC-1	EBV+, KSHV+ BCBL-derived B-cell line
BCBL	Body cavity based lymphoma
BJAB	Burkitt lymphoma-derived B-cell line
BL	Burkitt Lymphoma
CAT	Chloramphenicol acetyl-transferase
cDNA	Complementary DNA
CIP	Calf intestinal phosphatase
CV-1	African green monkey kidney-derived cell line
DAPI	4'-6-Diamidino-2-phenylindole
DBP	DNA-binding protein
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl-sulfoxide
DNA	Deoxynucleic acid

DUB	Deubiquitinase
DTT	Dithiothreitol
E	Early class of lytic transcription
EDTA	Ethyl-diamine tetra-acetic acid
EBV	Epstein-Barr virus
EDTA	Ethyl-diamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-Linked Immunosorbant Assay
ER	Endoplasmic reticulum
FLICE	Fas-associated death domain-like interleukin-1beta-converting enzyme
FLIP	Fas-associated death domain-like interleukin-1beta-converting enzyme-inhibitory protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gB	glycoprotein B
GST	Glutathione S-transferase
GRP-78	glucose regulated protein 78
hCMV	Human cytomegalovirus
HDAC	Histone de-acetylase

HEK-293	Human endothelial kidney- 293 cells
HeLa	Human liver-derived cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HHV8	Human herpesvirus 8 (KSHV)
IE	Immediate early class of lytic transcription
IL	Interleukin
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IsoT	Isopeptidase T
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KS	Kaposi's Sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late class of lytic transcription
LANA	Latency-associated nuclear antigen

MCD	Multicentric Castleman's disease
mCMV	Murine cytomegalovirus
mCP	minor Capsid Protein
MHV-68	Murine herpesvirus-68
mRNA	Messenger RNA
MTA	mRNA transport and accumulation protein
NERPRC	New England Regional Primate Research Center
nt	Nucleotide of DNA or RNA
ORF	Open reading frame
ORPRC	Oregon Regional Primate Research Center
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PRV	Pseudorabies Virus
RACE	Rapid amplification of cDNA ends
RIPA	Radio-immunoprecipitation assay

RhF	Rhesus fibroblast cell line
RNA	Ribonucleic acid
RRE	RTA-responsive element
RRV	Rhesus monkey rhadinovirus
RTA	Replication and transcription activator
RT-PCR	Reverse-trancriptase PCR
siRNA	Short interfering RNA
SDS	Sodium dodecyl sulfate
TBS	Tris Buffered Saline
TBSt	Tween-20, Tris-Buffered Saline
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TR	Terminal repeat
Ub-Al	Ubiquitin aldehyde
Ub	Ubiquitin
USP	Ubiquitin-specific protease
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

Vero	African green monkey kidney-derived cell line
vGPCR	viral G protein-coupled receptor
vIL-6	Viral interleukin-6
WT	Wild type

CHAPTER 1

INTRODUCTION

Kaposi's Sarcoma

Austro-Hungarian physician Moritz Kaposi worked for two years in the University of Vienna's syphilis clinic before deciding to change to dermatology. In the General Hospital's School of Dermatology and during the years 1868 and 1871 he treated 5 patients, all male and over 40 years of age, for what he called "*idiopathisches multiples pigmentsarcoma der haut*" or 'idiopathic multiple pigmented sarcoma of the skin (26). In his seminal 1872 paper he described the lesions as "Nodules the size of peas or hazelnuts, brown-red to blue-red in colour. They are either isolated or form groups; in the latter case, the central nodules of a plaque retrogress and generate a pitted, dark-pigmented depression." He notes that the lesions typically appear first on the feet and hands and later appear on the upper extremities, trunk, and face. "…Finally, identical nodules appear on the mucous linings of the larynx, trachea, stomach, intestines and liver. The disease is rapidly lethal, within two or three years." Dr. Kaposi determined it to be a systemic disease based on the simultaneous appearance of nodules on both feet and hands. The disease is now known as Kaposi's sarcoma or KS.

The KS lesion is composed mostly of proliferating spindle cells and is highly vascularized. The cellular lineage of these spindle cells is now thought to be endothelial or vascular endothelial in origin (55, 60, 127, 131). However, the lesion itself is complex and includes other cell types such as macrophages and inflammatory cells.

The clinical course of disease exhibits a wide spectrum which ranges from slow and indolent to rapid and fulminant with metastatic dissemination. Tissues involved include the skin (limbs, trunk and face), gastrointestinal tract (17, 29, 138) or respiratory tract (95, 114) . Today, the range of disease is classified as four distinct clinical types of Kaposi's sarcoma. Classic KS affects older men of Jewish and Mediterrenian backgrounds and represents the

type observed by Dr. Kaposi. The disease is mostly indolent, slow and characterized by lymphedema, and venous stasis followed by painful swelling of the hands and feet in the latter stages. Secondary malignancies develop in nearly thirty percent of Classic KS patients (56).

The second clinical type known as 'Endemic KS' mostly involves younger men and boys from tropical and southern Africa. Endemic KS presents with a far more aggressive disease and is associated with a high fatality rate (146). It also results in a widely varied neoplastic disease across central Africa and is characterized by involvement of multifocal mucocutaneous, lymphatic and visceral tissues (142, 147). Aggressive endemic forms of KS have been documented in children from central Africa (9, 166). A known risk factor for the Endemic subtype of KS is exposure to high levels of iron (167). Iron-rich soils in central Africa tightly correlate with a much higher incidence of endemic KS and the appearance of KS in the feet and lower legs, the areas in regular direct constant contact with the soil (167).

Iatrogenic or post-transplant KS is characterized by the onset of KS after solid organ transplants. Up to forty-five percent of patients will develop KS in the viscera in an average of 30 months post-transplantation (122). The development of post-transplant KS carries a poor prognosis (63, 122). Treatment includes altering or discontinuing the immuno-suppresive therapy and chemotherapeutics like paclitaxel (Taxol) (59, 120).

The advent of the AIDS epidemic also brought a sharp increase of KS cases particularly among HIV-infected homosexual men. The fourth and most widespread form of the disease is knows as Epidemic or AIDS-related KS. Epidemic KS is the most prevalent neoplasia in the HIV-positive population and is now recognized as an AIDS-defining illness, particularly in homosexual men. Up to fifteen percent of HIV-positive homosexual males

will develop AIDS-related KS (16). Epidemic KS is also the most aggressive form of KS, achieving a high degree of occurrence in heterosexual adults in developing nations like Africa and lower occurrence in children (16).

Kaposi's Sarcoma-Associated Herpesvirus

The exponential increase in KS cases during the 1980s suggested that KS could be transmitted by an infectious agent. Bacterial (74), chemical (115) as well as viral (71-73, 119, 152) culprits were considered including human cytomegalovirus (hCMV) and HIV.

Identification of the actual infectious agent was first accurately reported by Chang, et al in 1994 (41). Using representational difference analysis they found herpes-like sequences in KS biopsies that were specific to KS tumors when compared to normal tissues. The sequences were most closely related to Herpesvirus Saimiri (HVS) a gammaherpesvirus that induces lymphoma in New World monkeys and less so to Epstein-Barr Virus (EBV). Based on the limited homology to herpesviruses the authors proposed that the causative agent of KS represented a herpesvirus that was related, but distinct, from previously known herpesviruses. This etiologic agent of KS was named Kaposi's Sarcoma Associated Herpesvirus, or human herpesvirus eight (HHV8).

KSHV-related diseases

KSHV has since been shown to be present in all forms of KS lesions (21). More recently KSHV has also been implicated as the etiologic agent of two lymphoproliferative diseases; Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD).

Primary Effusion lymphoma or body cavity-based lymphomas (BCBL) have been shown to be ninety-eight percent infected with KSHV (39). EBV is usually also present although co-infection is not essential and the role of EBV in the development of PEL is not

clear (34). PEL is a non-Hodgkin's, diffuse-large B-cell lymphoma of clonal origin. It is characterized by liquid growth in the visceral cavities of the pleura, peritoneum and pericardium. An HIV-positive status and homosexual behaviors are two prominent risk factors. Upon development of PEL most patients face a negative prognosis, with a mean survival time between three to six months post-diagnosis (42).

Multicentric Castleman's Disease (MCD) is a multifocal hyperplasia of polyclonal origin that is clinically characterized by systemic manifestations. These include fever, weakness, generalized lymphadenopathy, and hyper-gamma-globulinemia which are due in part to raised serum concentrations of interleukin 6 (75, 117). Exacerbation of the disease correlates with an increase in KSHV viral loads in peripheral blood mononuclear cells (PBMCs). Patients also suffer from cytopenias, rashes, and recurrent infections and some have been reported to develop non-Hodgkin lymphoma. KSHV-associated MCD is in most cases refractory to corticosteroids and chemotherapy. The resistance of MCD to systemic treatments makes MCD potentially lethal.

Epidemiology

KSHV seroprevalence varies widely and is not as widespread as that of other herpesviruses. In African regions where KSHV is endemic, fifty to seventy percent of individuals may be positive (76, 135). The incidence of KS roughly correlates with seroprevalence of the disease. However, gross disparities between seroprevalence and incidence in certain populations point to the importance of other potential aggravating factors in the progression from KSHV infection to KS (148).

Although there are data documenting the sexual, horizontal, and parental transmission of KSHV, evidence defining the mode of KSHV transmission has thus far been elusive. Men who have sex with men have a markedly increased prevalence of infections which further

increases with the number of male partners (35, 38). Pre-existing sexually transmitted diseases (STDs) and intravenous drug use increase the likelyhood of infection. The risk of developing KS is also significantly increased with HIV co-infection (109). These data suggest that sexual transmission may be a viable route of infection. However, heterosexual couples have a low degree of prevalence (143) that is thought to be due to KSHV being poorly infectious (25, 78). Interestingly, saliva seems to be a more infectious body fluid than semen. KSHV viral load in semen from HIV-positive or HIV-negative patients is low (84) while viral loads in saliva can be quite high (121). Moreover, transmission via salivary secretions has been documented (154).

Areas with endemic KSHV produce cases where virus shedding occurs from the oropharynx of immunocompetent and immunocompromised men and women alike (36, 37, 151). In these areas transfusion recipients show an increased risk of KSHV transmission (33, 83). In addition, transmission of KSHV through solid organ transplants has also been documented (12, 106). Transmission through exposure to saliva has been shown between mother and child, and between siblings (108, 125).

Disease Manifestations of Human Herpesviruses

Herpesviruses are enveloped DNA viruses with large coding capacities ranging from 100 to over 200 open reading frames. These viruses establish slow-progressing, life-long infections in their host, thus their name herpes from the Greek *herpein* meaning “to creep”. Herpesviruses have been classified into three families, Alpha, Beta and Gamma (Fig. 1). The Alpha Herpesviruses include herpes simplex type-1 (HSV-1), herpes simplex type-2 (HSV-2) and Varicella zoster virus (VZV). HSV-1 and HSV-2 are responsible for herpetic lesions of the oral mucosa and genitalia, respectively. Varicella zoster is the etiologic agent of chicken

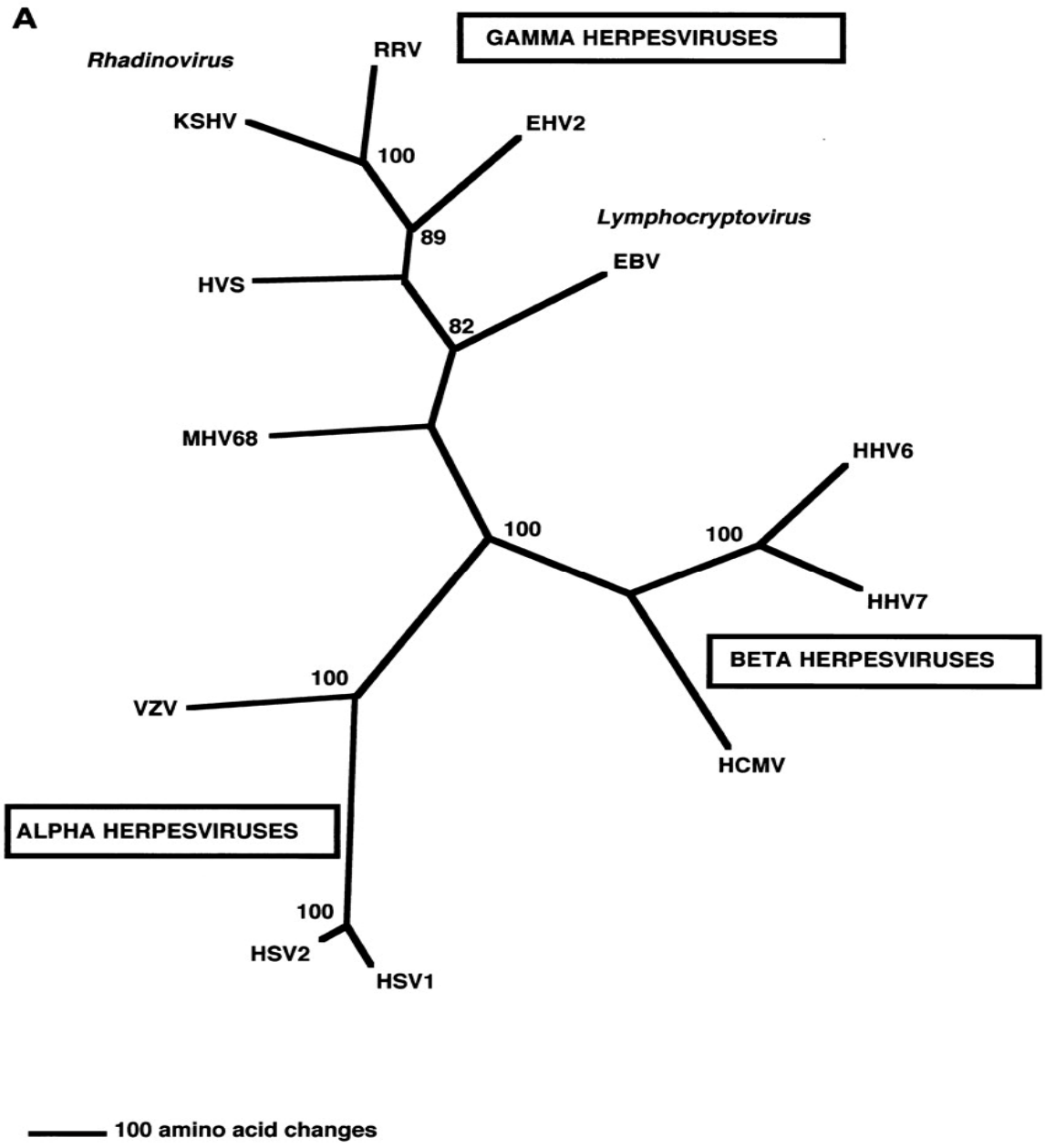

Figure 1. Classification of herpesviruses. A phylogenetic tree depicting the alpha, beta, and gamma subfamilies of herpesviruses. The phylogram is unrooted and was constructed by parsimony analysis using the viral DNA polymerase gene and the neighbor-joining method. The scale shown below the tree represents number of amino acid changes. (Taken from Damania and Jung (46).)

pox as well as shingles, which is associated with reactivation of VZV. The prototype for alpha-herpesviruses, HSV-1, infects epithelial cells on the mouth and lips and travels to the dorsal root ganglion where it can establish a latent infection. From there it periodically reactivates and infects innervated epithelial cells.

Human Cytomegalovirus or HCMV causes disease in small children, unborn babies and transplant recipients. HCMV belongs to the beta family of herpesviruses as does HHV-6 and HHV-7. HCMV infection is a leading cause of birth defects and a major cause of morbidity and mortality in immune-compromised individuals (48, 66). HCMV prevalence can be found world-wide. Disease manifestations in immune-compromised patients include infectious mononucleosis, pneumonia, hepatitis, CNS involvement (Guillain-Barré syndrome) aseptic meningitis, and immunologic abnormalities. In contrast, the immune-competent host has a mostly asymptomatic infection.

Gamma-herpesviruses are further classified into two subclasses, the genus lymphocryptovirus (Gamma-1) and the genus rhadinovirus (Gamma-2). While KSHV belongs to the rhadinovirus or gamma-2 grouping of herpesviruses, Epstein-Barr Virus (EBV) is the archetypal lymphocryptovirus and can be found world-wide. EBV can cause a wide range of B-cell lymphoproliferative disorders, including Burkitt lymphoma (BL), classic Hodgkin lymphoma (HL), and lymphomas arising in immune-compromised individuals (post-transplantation and HIV-associated lymphoproliferative disorders (49, 161).

Lifecycle of Herpesviruses: Latency and Lytic replication

Herpesviruses are distinguished from other viruses by their inherent ability to exist in bimodal replicative states. The herpesviral characteristic state is that of latency which alternates with the state of productive lytic replication. Herpesviral infection of the host cell is initiated by specific interactions between viral envelope proteins and cell surface receptors as well as non-specific binding to heparin sulfate moieties. In the case of KSHV, integrin alpha3beta5 has been identified as a cellular receptor (3). The initial binding of virus to receptor triggers fusion of the viral and plasma membranes which liberates the virion into the cytoplasm. Once inside the cell, the virion proteins are shed allowing the linear viral genome to circularize and travel to the nucleus. After nuclear entry the viral genome can go into either lytic replication or latency.

Lytic Replication

Productive lytic replication is characterized by replication of viral genomes, followed by assembly and release of viral progeny resulting in cell lysis. However, these processes occur in a succession of steps dictated by a highly-coordinated set of transcriptional waves known as immediate early (IE), early (E) and late (L). The temporal regulation of transcription ensures that the IE, E, and L proteins required at each step are available when needed. Moreover, uncontrolled expression of all viral ORFs would be massively inefficient and counterproductive to viral replication and survival.

The initial or IE class of transcription is limited to viral proteins that transactivate other viral promoters thereby setting the stage for the next wave of cellular and viral transcription. Early genes are mostly involved in viral DNA replication and include the six conserved core replication proteins: DNA polymerase, processivity factor, helicase, primase,

primase-associated factor, and a ssDNA binding protein. These in addition to the IE protein KSHV RTA and K8 are required for the replication of the viral genome from two distinct origins of lytic replication (*ori-lyt*) (AuCoin-04Virol). The protein complexes formed on these *ori-lyts* mediate replication via the rolling circle mode characteristic of herpesviruses.

After the onset of viral DNA replication, the L genes are transcribed which code for the structural components of progeny virions. Once L proteins have been expressed the nascent virions are assembled in the nucleus, and start to egress. During egress the capsids will acquire an array of viral and host proteins known as the tegument, which decorate the outer capsid. The capsid and tegument then acquire an outer membrane embedded with viral glycoproteins. Mature virions reach the plasma membrane and are released to infect neighboring cells.

Latency

The latent state is defined by the expression of a very limited complement of viral proteins. This small group of viral gene products protects the virus from antigen presentation and the immune response, while also stimulating cell cycle progression. Latency transcripts include LANA, viral cyclin (vCyclin), a viral FLICE inhibitor (vFLIP), viral interferon regulatory factors 1 and 3 (vIRF-1 and vIRF-3) in endothelial and B cells, respectively (57, 126), K15 (140), kaposin or K12 (132), and the viral microRNAs (31). The viral genome persists as a circle termed an episome which is tethered to the host chromosome. The latency-associated nuclear antigen (LANA) binds the viral genome at its terminal repeats (10) and the host chromosome (124) via histones H2A/H2B (11). This two-fold interaction of LANA with the viral TR and the host chromosome ensures that the viral episome segregates to daughter

cells after mitosis. During latency the viral genome is replicated by the cellular machinery and LANA is essential in this respect as well (141).

Reactivation

Certain stimuli can trigger latent cells to undergo reactivation. Reactivation generally results in the viral genome entering the lytic phase of replication, although at time this may be abortive rather than complete replication. Latency allows for viral persistence and evasion of immune surveillance, but reactivation is viral replication and dissemination to new cells and tissues. Although latent virus can be reactivated *in vitro* by phorbol esters (TPA) (105) or histone de-acetylase (HDAC) inhibitors (N-butyrate), the *in vivo* factors that act as triggers of reactivation remain elusive. Reactivation is enhanced by immune-deficiencies and hypoxia may be another such factor. Expression of the lytic switch protein, RTA, is induced under hypoxic conditions (30, 47, 81). This finding correlates very well with the fact that classic KS develops first in the feet and hands, sites where older patients frequently show poor circulation. Solid tumors also face the prospect of hypoxia as the tumor grows and requires more nutrients and oxygen. It is possible that in this scenario hypoxia induces reactivation of some KSHV-infected cells and may contribute to the neoplastic process.

The Viral Genome

The KSHV genome resembles a typical herpesvirus genome in its large size of nearly 140-150 kilobase pairs (kb). However, unlike most herpesviruses there is a single unique-long region and no unique-short region. The unique-long region is flanked by 20-35 kb of terminal repeat (TR) sequences. The terminal repeats consist of high G+C content, 801 bp repeats (130). Conserved, core replication genes are concentrated towards the center of the genome where there is less recombination-mediated variability. Less conserved and unique

genes tend to be located towards the ends where rates of recombination are higher (Fig. 2). There are close to eighty open reading frames encoded by KSHV. The functions for most ORFs have been predicted from sequence homology to proteins from other herpesviruses and most ORFs have had some empirically-confirmed function. Still, some ORFs remain to be functionally described. The KSHV genome includes core herpesviral genes, KSHV-specific genes and genes that are homologous to cellular gene. These viral homologs have most likely been "stolen" from the host in what is referred to as "molecular piracy" (111). The virus has altered these genes in order to subvert host control mechanisms and thus modify the cellular environment to suit its particular needs. Cellular homologs in KSHV include a viral cyclin (vCyclin), viral FLICE inhibitor (vFLIP), viral bcl-2 (vbcl-2), viral IL-6 (vIL-6), four viral IRFs (vIRFs), two viral MIPs (vMIP1 and 2), and a constitutively active G protein-coupled receptor (vGPCR). They mediate viral-induced disruption of cell cycle progression and cellular signaling pathways, and can also down-modulate the immune response.

Model Systems

Early *in vitro* studies of KSHV have been carried out in a variety of cell lines originating from PEL malignancies. PEL-derived B-cell lines maintain the viral episome indefinitely and are one hundred percent infected with KSHV (8, 21, 32, 68, 82, 91). The establishment of these cell lines requires transformation for *in vitro* adaptation. In these cells latency is the norm. However, a small subset of cells (about five percent)

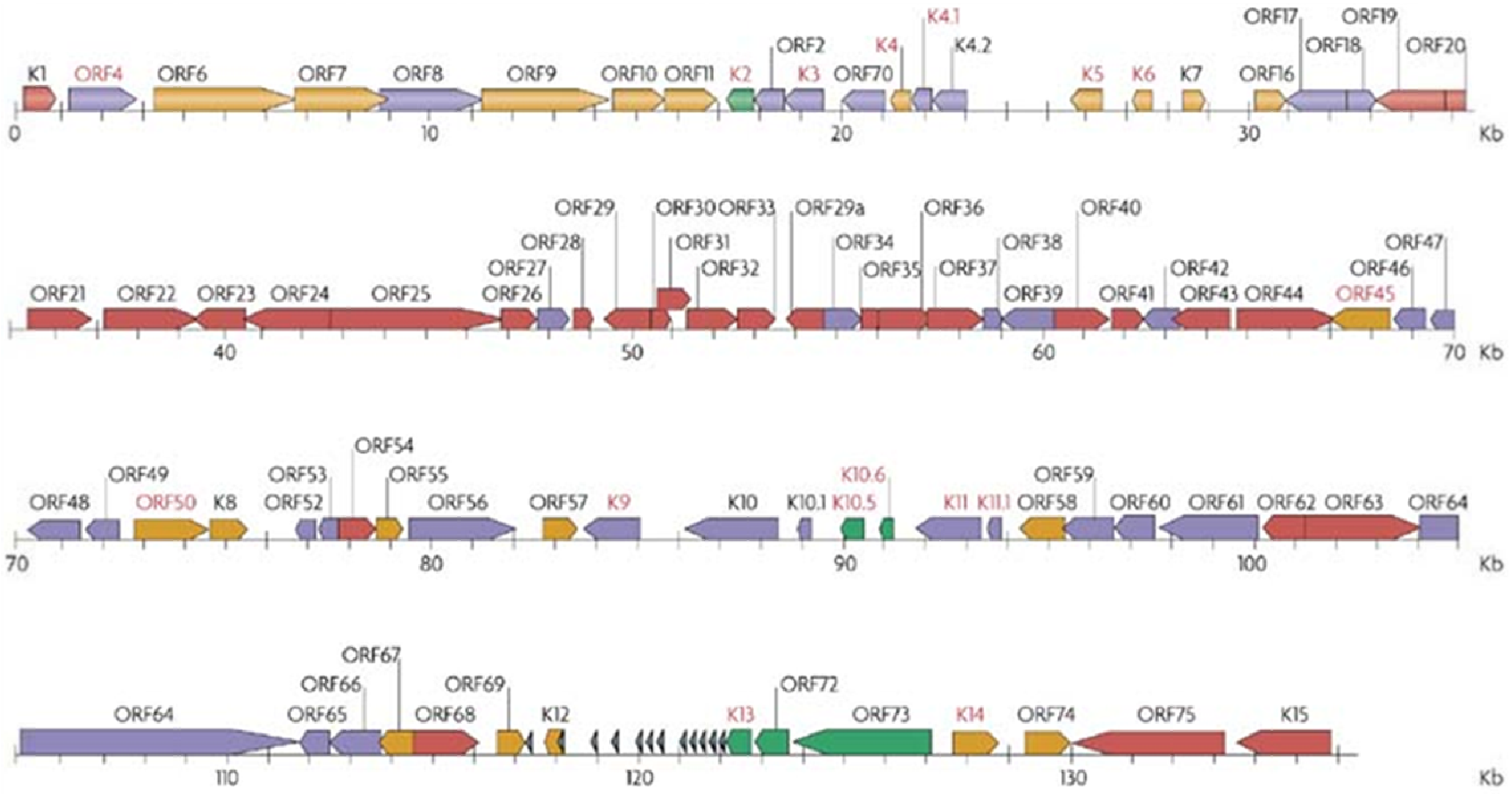

Figure 2. Schematic representation of the KSHV genome. Proteins expressed during latency are colored green. Lytic proteins are color-coded as follows: immediate early (IE) is yellow, early (E) in orange, and late (L) in red. The microRNAs encoded by in the genome are designated with gray arrowheads. ORFs involved in immune evasion are labeled in red font. (Taken from Coscoy, 2007)

spontaneously enter productive lytic replication. Lytic reactivation can be induced in these cell lines by ectopic expression of RTA or chemical treatments such as phobol ester or HDAC inhibitors (104, 128). The proportion of cells that are induced is nonetheless quite low, peaking at twenty percent. Thus, the study of KSHV reactivation and pathogenesis in this system has been performed in the presence of a high degree of background latency. Likewise, the study of latency is hampered by the small degree of spontaneous lytic reactivation that occurs with these cell lines. Moreover, because they are already transformed, this system cannot help to delineate the mechanism of KSHV-induced transformation.

Endothelial cells (EC), believed to be the primary target of *de novo* infection and the precursors of spindle cells, have been shown to be infected and transformed by KSHV in vitro (7, 65). These cells also displayed the classic markers of KS and the development of the characteristic spindle cell morphology. However, immortalized ECs as well as primary ECs infected *in vitro* quickly switch to a latent state and shed the viral episome making this system inadequate for the study of lytic replication and transformation. B-cell lines either established or primary can be infected *in vitro* but cannot maintain the episome after extended passages (13, 18). Intriguingly, cell lines established from KS lesions are also unable to maintain the viral episome (5, 6, 14, 98, 144).

Limits in existing cell culture systems have hampered the study of viral replication and transformation. The development of a bacterial artificial chromosome (BAC) system for KSHV (163) has proved to be useful in allowing the study of individual ORFs in the context of the whole viral genome (94, 159, 160). In addition, this system makes possible the engineering of viral genomes that generate live recombinant viruses. With live recombinant

viruses we are now able to explore the contributions of individual genes to the biology of the entire virus.

Previously, studies of individual ORFs were limited to cloning and over-expressing ORFs in the absence of the viral genome. This approach can delineate the effects of individual proteins only if they are capable of acting in the absence of other viral proteins. Cooperative actions of several ORFs or all ORFs encoded by the viral genome are cumbersome and inefficient to study this way. Moreover, since these experiments are also confined to tissue culture systems, these types of experiments cannot predict how the gene or genes under study will affect host pathogenesis.

Model animal systems for KSHV are lacking. The extent of animal use for KSHV research has been limited to recombinant inbred mice. Xenograft injection of KSHV infected B-cells (BCBL-1 or BCP-1) into NOD/SCID and PEL cells into C.B.17 SCID mice (21, 45, 123, 145) have been documented. In addition, human cells or tissues have been transplantated into mice and have been shown to develop human, KSHV-specific antibodies and identified CD34+ hematopoietic progenitor cells (HPC) as a possible reservoir for KSHV infection (44, 58, 67, 118, 158). Daily systemic treatment with Azidothymidine (AZT) and IFN-alpha curtailed viral replication and increased mean survival times in these animals.

Other animal studies have managed to recapitulate several key features of the KS tumor. Normal mouse bone marrow-derived endothelial cells (mECs) transfected with the KSHV BAC were injected into nude mice. The xenograft generated vascularized spindle cell sarcomas which result in KS-like tumors with overexpression of VEGF and Angiopoietin ligands and receptors. In spite of this, the infection was nonproductive and reverted to nontumorgenicity once the viral genome was lost (113).

Finally, although progress has been made in the *in vitro* and *in vivo* fronts, we are still lacking a robust animal model system for the study of KSHV-induced tumorgenesis. Mice, in addition to being inbred and thus not representative of a heterogeneous human population, are only distantly related to humans. A better, more closely related animal system like non-human primate would be best, although significantly more costly.

RRV

Another member of the gamma-2 or rhadinovirus sub-family is Rhesus monkey rhadinovirus (RRV). Immunocompromised rhesus macaques infected with RRV develop diseases that resemble KSHV-associated multicentric Castleman's disease and non-Hodgkin's lymphoma. Rhesus macaques are naturally infected with RRV.

In 1997, the New England Primate Research Center (NEPRC) and the Oregon Regional Primate Research Center (ORPRC) practically simultaneously reported the discovery of two distinct strains of a monkey rhadinovirus termed rhesus monkey rhadinovirus or RRV (strains H26-95 and 17577, respectively) (15, 53). The H26-95 strain was isolated from primary rhesus fibroblasts exhibiting cytopathic effects (CPE) after co-culture with PBMCs from healthy rhesus macaques. Strain 17577 was isolated from an SIV-infected macaque presenting with a lymphoproliferative disease. Both strains were fully sequenced less than three years after their discovery (4, 136).

In culture, rhesus fibroblast (RhF) infection with RRV is completely lytic. After *de novo* infection, the virus is replicated and progeny virus is released until every cell in culture is killed. RRV infection generates CPE and is susceptible to plaque assays since it kills RhF. Virus production and titers are high compared to other gamma-herpesviruses reaching up to 10^6-10^7 plaque forming units (PFU) per ml. The high rate of lytic replication also allows the

manipulation of the viral genome and establishment of different assays to measure viral replication (54). The advent of BAC-assisted RRV genome (61) now allows more convenient manipulation of the viral genome for further study.

As discussed previously, efforts to investigate KSHV pathogenesis have been hampered by the virus' slow growth kinetics, limited infectivity in cell culture and lack of an animal model. RRV, in contrast can grow to high titers in cell culture of RhF and naturally infects rhesus macaques. Moreover, The RRV genome shows a high degree of homology and colinearity with the KSHV genome (Fig. 3.). Every gene in RRV is represented in KSHV. Finally, SIV plus RRV co-infection recapitulates the lymphoproliferative diseases seen in AIDS patients. Taken together these finding indicate that RRV provides a suitable animal system for the study of KSHV pathogenesis.

KSHV viral proteins involved in the modulation of the lytic cycle

KSHV RTA/Orf50

The KSHV RTA/ORF50 encodes a replication and transcription activator. The RTA protein is one of several core herpesviral proteins that is conserved across the gammaherpesviridae. The importance of this protein for the virus is evidenced in the high degree of homology between related viruses. The degree of conservation is such that RTA protein from KSHV can induce RRV RTA-responsive promoters and vice-versa making them functionally interchangeable.

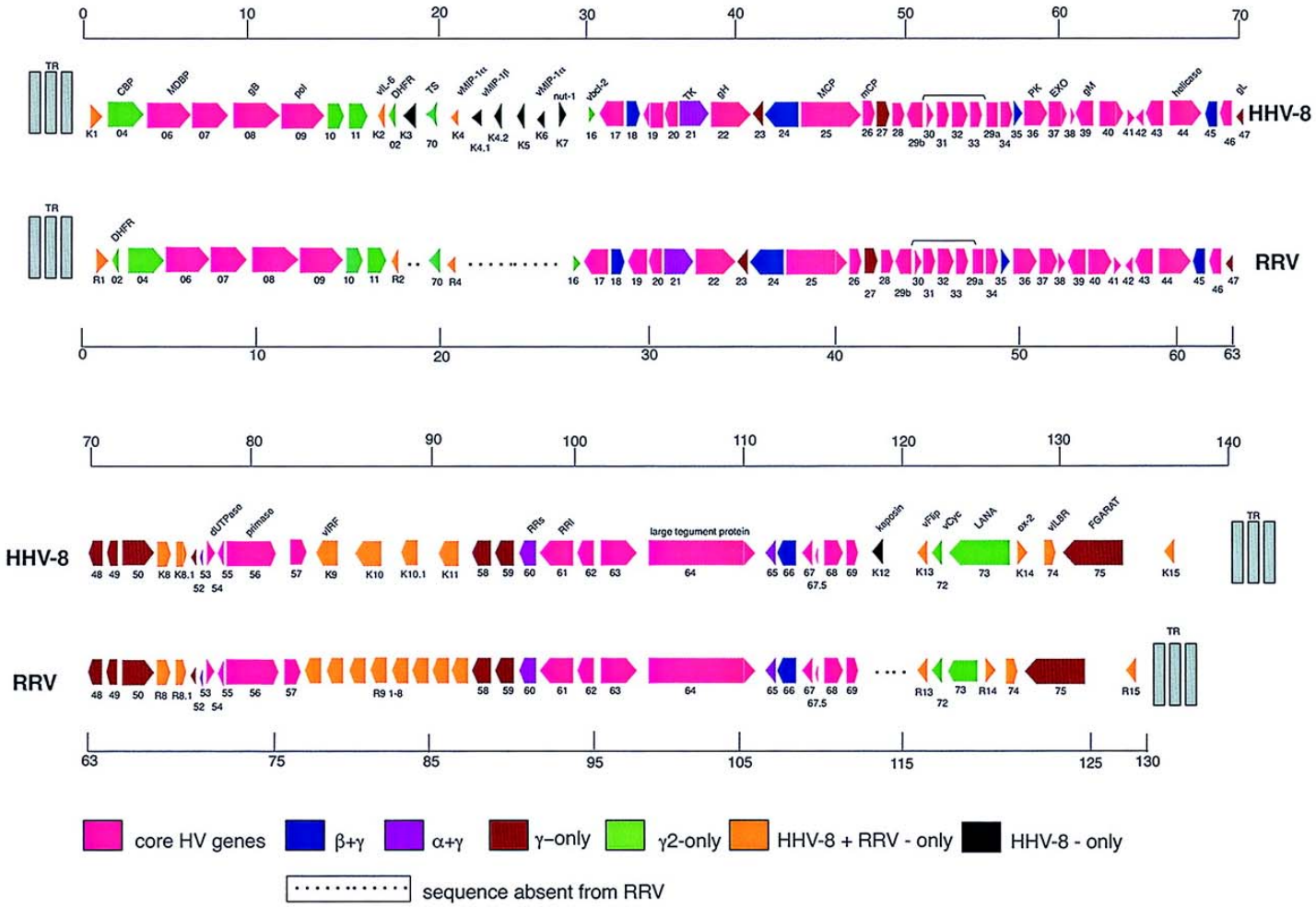

Figure 3. Alignment of KSHV and RRV26-95 genomes. Color coding denotes ORFs in KSHV and RRV26-95 conserved in the indicated herpesvirus subfamilies or subgroups. The arrow-shaped side of each ORF signifies the 3' end whereas the square side corresponds to the 5'end. The ORFs are not drawn to scale. (Taken from Alexander, 2000)

The RTA protein is multifunctional and is termed the lytic switch protein due to its ability to single-handedly induce the lytic replication cycle (105, 149). Indeed, RTA has been shown to be necessary and sufficient for lytic induction. Recently, a KSHV-BAC recombinant virus lacking ORF50 (BAC36Δ50) was reported to be tightly latent (159).

The ability of RTA to single-handedly activate the entire lytic cascade depends on two separate mechanisms. RTA can directly bind promoters through RTA-responsive elements (RREs) to induce transcription from them (101, 103). This is true for Orf57 (MTA), K8 (K-bZIP, ZTA) (103), K12 (kaposin), and polyandelytated nuclear RNA (Pan) (40). In all cases electrophoretic mobility shift assays (EMSA) have shown that transcription from these promoters requires direct binding by RTA. A second mechanism of transcriptional activation involves binding to cellular components. RTA can bind to recombination signal-binding protein Jkappa (RBP-Jk). RBP-Jk binding sites in the targeted promoters recruit an RTA-RBP-Jk complex to the promoter where RTA can induce transcription (100). Another cellular RTA binding partner is the transcription factor CCAAT/enhancer-binding protein-alpha (CEPB-α). Similar to RBP-Jk, CEPB-α binds both K8 and RTA and upregulates transcription from the RTA, Orf57 and K8 promoters. Again, EMSA studies showed that CEPB-α recruits RTA and K8 to CEPB-α-binding sites in these promoters and that binding is required for optimal induction (156). Additional binding partners of RTA during transcriptional activation include Oct-1, Sp-1, c-Jun, and CREB-binding protein (CBP) (79, 133, 162). Other promoters induced by RTA include K1, ORF6, ORF9, vIL-6, K5, K9, Orf59, thymidine kinase (TK), vGPCR, K14, LANA and ORF50 (24, 50, 80, 87, 96, 104, 105, 110, 133).

KSHV ORF49

ORF49 is an open reading frame in the RTA locus that is encoded on the complementary strand. It is flanked by a short exon and the second long exon encoding the RTA gene. The ORF49 gene shows limited sequence homology to the EBV BRRF1 gene. The EBV BRRF1 is also contained within the EBV RTA homolog, BRLF-1 locus. These loci are collinear and are subject to a splicing event resulting in the removal of the ORF49 and BRRF-1 sequences from the ORF50 and BRLF-1 transcripts, respectively (104, 105, 137) (Fig. 4.). The BRRF-1transcript was detected in Raji and Akata cells treated with TPA/Na-butyrate or anti-IgG respectively. The BRRF-1 protein was found predominantly in the nucleus of transfected cells.

KSHV ORF64

The gene encoded by KSHV ORF64 is predicted to be a large tegument protein due to homology to other herpesviruses. It is conserved across the alpha, beta and gammaherpesviridae and is considered a core herpesviral protein. The ORF64 gene is the longest single sequence in the genome with close to 8kb of coding sequence. The predicted molecular weight of this protein is approximately 300kDa. Thus far, one study used yeast-two-hybrid and co-immunoprecipitations (co-IP) to show that ORF64 interacts with itself, with ORFs21, 45, and 63 plus glycoproteins gB, gH, and gM (129). These data echo the findings of Zhu et al (165) where ORF64 was found to be tightly associated with the viral capsid. Interestingly, this group found ORF64 to be sensitive to protease

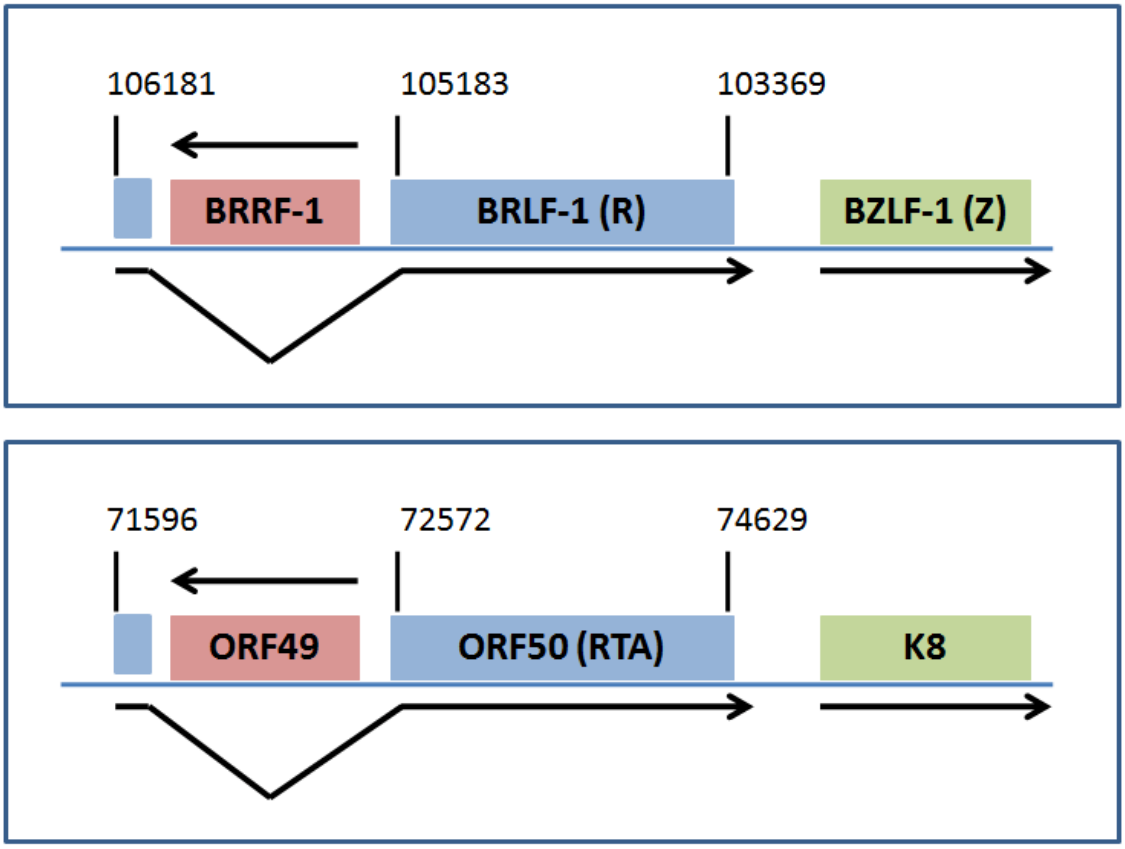

Figure 4. Comparison of the BRLF-1 and RTA loci of EBV and KSHV. The upper panel shows the EBV BRLF-1 locus gene layout. BRRF-1 is in opposite orientation to BRLF-1 and adjacent. The lower panel represents the homologous locus in KSHV encoding RTA (ORF50) and ORF49. The overall gene organization and splice patterns in these loci are well conserved. (Nucleotide positions for EBV and KSHV are from B95-8 and NC_003409 strains, respectively)

treatment of the virion. This finding suggests that ORF64 is in contact with the capsid and the envelope of KSHV virions where it is exposed to protease and bind glycoproteins. The significance of these findings is currently under investigation.

ORF64 and its homologues in all the herpesviruses have predicted deubiquitinase (DUB) domains encoded in their N-termini. DUB activity has now been identified in several herpesviruses (134). In Herpes Simplex virus-1 (HSV-1), and Pseudorabies virus (PRV) (α-subfamily) the UL36 gene encodes a multifunctional, large tegument protein that are DUBs. In human Cytomegalovirus (β-subfamily), the homologous UL48 protein also encodes DUB activity (155).

Although the targets of the herpesviral deubiquitinases remain to be discovered, studies of HSV-1 mutants have implicated the HSV DUB in virion transport through microtubules (139), release of the viral genome into the nucleus (1, 89), tegumentation and envelopment (52) as well as virion egress (112). A DUB-null mutant of HSV UL36 displayed a 3-log decrease in titers and a 50% reduction of egressing capsids from neurons (97) suggesting that DUB activity plays an important role in lytic replication. Null mutations for PRV UL36 have been shown to be lethal *in vitro* (107) and delay neuroinvasion in the mouse model (23). Moreover, specifically mutating the DUB catalytic domain reduces titer and plaque size (22). Finally, it was recently shown that a DUB mutant of Marek's disease virus was severely impaired in its ability to induce lymphomagenesis (86).

The NFκB pathway

Nuclear factor (NF)-κB is an evolutionarily conserved signaling molecule that plays a central role in the regulation of a variety of cellular processes including the immune system (70) (99) (19). The NF-κB family is made up of p50/p105 (NF-κB1), p52/p100 (NF-κB2), p65 (RelA), RelB, and c-Rel. In their steady state they form homo- or heterodimers in the cytoplasm. The inhibitor of κB (IκB) proteins binds to and prevents NF-κB translocation to the nucleus. Signaling through NF-κB follows two parallel pathways, the canonical or non-canonical (19). Pro-inflammatory cytokines, pathogens or antigenic peptides can activate this pathway. Once induced, the classical pathway activates the β subunit of the IκB kinase (IKK) complex, which can phosphorylate IκB proteins and trigger their ubiquitination and degradation.

The non-canonical or alternative pathway, is induced by activation of the α subunit (IKKα), which in turn phosphorylates p100. IκB proteins are phosphorylated and subsequently ubiquitinated through lysine 48 (K48) by the Skp1–Cullin1– F-box (SCF)/-transducin repeat-containing protein (TrCP) E3-ligase complex.

Poly-ubiquitination targets IκB molecules for processing by the preteosome into p50, p65 and c-Rel. The resulting subunits form NF-κB heterodimers that translocate to the nucleus. Once in the nucleus, the heterodimers bind to NF-κB-responsive promoters of target genes.

There are seven members of the IκB family: IκBα, IκBβ, IκBγ, IκBε, BCL-3, p100 and p105 (19). IκB degradation is induced by phosphorylated and thus activated, IKK complex. The IKK complex includes two kinase subunits, IKKα (IKK1) and IKKβ (IKK2),

and IKKγ, or NEMO (NF-κB essential modifier), a regulatory subunit (19, 69). IKKγ, considered the 'molecular switch of NF-κB activation', has been shown to be non-destructively regulated through ubiquitination through different lysine residues (2, 85, 164).

The TNF receptor (TNFR)-associated factor 6 (TRAF-6) is a lysine-63 (K63)-specific E3 ligase which is required for IKK complex function in *in vitro* assays (43). TRAF-6 mediated ubiquitination of IKKγ is essential for IL-1 receptor (IL-1R) and Toll-like receptor (TLR) signaling (51, 90). TRAF-2 is another K63-specific E3 ligase that mediates polyubiquitination of RIP (receptor-interacting protein) (157) which plays a pivotal role in receptor complex formation after TNF-α-stimulation. TRAF2/TRAF5 doubly deficient mice (150) display marked inhibition of NF-κB activation during TNF receptor (TNFR) induction. Thus, TRAFs 2 and 5 could function in TNF signaling as analogs to TRAF6 during IL-1 activation. IL-1 induction causes TRAF6 binding to TGF-β-activated kinase 1 (TAK1) which activates the NFκ-B pathway (116). TAK1 kinase activity is regulated by TAK1-binding (TAB) proteins TAB2 and TAB3. TABs recognize proteins ubiquitinated by TRAF2 and TRAF6 (90).

CYLD is a cellular, K63-specific DUB capable of inhibiting NF-κB signaling (28, 93, 153). Mutations in the CYLD gene result in an autosomal-dominant disease familial cylindromatosis. The disease is characterized by benign tumors arising from hair follicles or eccrine gland cell types. NF-κB activation induces CYLD expression which inhibits IKK activity (88). The mechanism of NF-κB signaling inhibition by CYLD involves the cleavage of K63-linked Ub chains from TRAF2, TRAF6 and IKKγ.

Another cellular DUB that interferes with TNF-α-induced NF-κB responses is A20 (20). A20 inhibits IKK activation by cleaving K63-linked ubiquitin chains from RIP and

TRAF6. The deubiquitination of RIP and TRAF6 block signaling to NF-κB (20, 157). A K48-specific Ub E3 ligase is also encoded by A20. The E3 module can catalyze the K48-linked poly-ubiquitination of RIP (157). Cezanne is another DUB that may regulate TRAF6 (62). Cezanne blocks NF-κB signaling possibly through IKK deubiquitination.

Various conflicting data on the effects of KSHV infection on the NF-κB activation have been reported. Inhibition of NF-κB with Bay 11-7082 (Bay-11) increased levels of lytic reactivation in PEL cells (27). Additionally, activation of KSHV lytic promoters is countered by p65 expression. In latently infected cells v-FLIP expression is high and has been shown to induce NF-κB activation (64) (102). In contrast, treatment of PEL cells with Bay-11 results in an induction of apoptosis and suggests that NF-κB activity is required for PEL cell survival (92). Moreover, inhibition of the NFκB pathway in human fibroblasts does not increase lytic reactivation and activated NFκB has been reported during lytic replication in vous cell types (77).

OBJECTIVES

1) Characterization of KSHV ORF49

There have been no previous studies on KSHV ORF49 or its role in the viral life cycle. The initial study of KSHV ORF49 aimed to characterize both transcript and protein. The kinetics of KSHV Orf49 transcription and the 5' and 3' ends of the transcript (RACE) were identified. The properties and function of the encoded protein were investigated as well as the contribution to lytic replication.

2) Characterization of KSHV ORF64

KSHV ORF64 has been shown to be present in the tegument of virions and to associate with several tegument and glycoproteins but has not been ascribed a function. We aimed to identify potential roles of KSHV ORF64 in the viral life cycle. DUB enzymatic activity was identified in the N-terminus of KSHV ORF64 by a number of different assays. The role of virus-encoded DUB activity in the context of lytic replication was assessed.

3) KSHV ORF64 DUB activity and signaling

The robust DUB activity encoded by KSHV Orf64 suggests a large pool of potential protein targets of Orf64-mediated deubiquitination. Since several signaling pathways pertinent to viral infection are regulated through ubiquitination, we have tried to identify potential targets of the KSHV-encoded DUB.

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CHAPTER TWO

IDENTIFICATION AND CHARACTERIZATION OF THE KSHV ORF49 PROTEIN

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). KS is the most common neoplasm among HIV-positive individuals. Like other herpesviruses, KSHV is able to establish a predominantly latent, life-long infection in its host. The KSHV lytic cycle can be triggered by a number of stimuli that induce the expression of the key lytic switch protein, the replication and transcription activator (RTA) encoded by Orf50. The expression of Rta is necessary and sufficient to trigger the full lytic program resulting in the ordered expression of viral proteins, release of viral progeny and host cell death. We have characterized an unknown open reading frame, Orf49, which lies adjacent and in the opposite orientation to Orf50. Orf49 is expressed during the KSHV lytic cycle and shows early transcription kinetics.

We have mapped the 5' and 3' ends of the unspliced Orf49 transcript, which encodes a 30kD protein that is localized to both the nucleus and the cytoplasm. Interestingly, we found that Orf49 was able to cooperate with Rta to activate several KSHV lytic promoters containing AP-1 sites. The Orf49-encoded protein was also able to induce transcriptional activation through c-Jun but not ATF1, ATF2, or CREB transcription factors. We found that Orf49 could induce phosphorylation and activation of the transcription factor, c-Jun, the Jun N-terminal kinase (JNK) and p38. Our data suggests that Orf49 functions to activate the JNK and p38 pathways during the KSHV lytic cycle.

INTRODUCTION

Kaposi's Sarcoma-associated Herpesvirus (KSHV) is etiologically linked to Kaposi's sarcoma (KS) (12, 17, 25) primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (3, 4). KS is the leading neoplasm in HIV- positive individuals. KSHV is the most recently described human herpes virus, identified in 1994 by Chang et al.(6) via representational difference analysis (RDA). A member of the gamma-subfamily of herpes viruses, KSHV is also the only known human rhadinovirus. Like other herpesviruses, KSHV can establish both latent and lytic infection in its host. The lytic program can be triggered by a number of stimuli that induce the expression of the lytic switch protein, the replication and transcription activator (RTA) encoded by Orf50 (38). The expression of RTA is necessary and sufficient to trigger the full lytic program (21, 22) resulting in the ordered expression of all viral proteins, release of viral progeny and host cell death. RTA/Orf50 initiates the lytic cascade by inducing the transcriptional activation of viral promoters like PAN, ORF6 (single stranded DNA binding protein), K1, K2 (vIL-6), K5, K6 (vMIP-1), K8 (MTA), K9 (vIRF), ORF57 (MTA), ORF50 (RTA), thymidine kinase, K12 (kaposin), K14 (vOX-2), and ORF74 (vGPCR) (5, 7, 9, 10, 16, 20, 21, 33, 37, 39, 44).

This wide range of promoter activation occurs through sequence-specific binding of Orf50 to DNA via RTA responsive elements (RREs) (5, 9, 20, 37) and binding to cellular transcription factors like C/EBP-α and RBPJ-κ (19, 41). Another protein expressed in the lytic cascade is K8 or K-bZIP, a structural and positional homologue of EBV ZTA. However, unlike EBV ZTA, K-bZIP is unable to activate lytic transcription from latency (30). K-bZIP shows strong trans-repression of viral ORF50 (15) and cellular p53 (28) promoters. SUMOylation of K8 mediates its repression (14).

Studies on KSHV pathogenesis have been limited to latently infected PEL-derived B cell lines like BCBL-1, BC-2, JSC-1, BC-3 or BC-1. In these cell lines, latency can be disrupted by the addition of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or histone de-acetylase (HDAC) inhibitor, sodium butyrate (24, 32). However, the efficiency of reactivation as measured by viral particle production, is very low, peaking at approximately twenty percent (42). Moreover, studies of KSHV latency are complicated by the fact that, at any time, ~5% of these cells are undergoing spontaneous lytic reactivation.

In an effort to enhance our understanding of KSHV pathogenesis, we focused our attention on Orf49, an uncharacterized open reading frame in the lytic switch locus. Orf49 is located adjacent to Orf50 and on the opposite strand. The gene encoded by Orf49 shows limited sequence homology to the EBV BRRF1 gene. EBV BRRF1 lies in the same relative orientation as KSHV Orf49 and is subject to a similar splicing event that omits the BRRF1 sequence from the EBV RTA transcript (34).

Here we describe the initial characterization of KSHV Orf49 and its encoded protein. We have identified the 5' and 3' ends of the Orf49 transcript using rapid amplification of cDNA ends (RACE) and have characterized its transcription kinetics. The Orf49-encoded protein by itself could not activate any of the KSHV promoters we tested by promoter reporter assays, but it was able to cooperate with KSHV Orf50 to activate a number of lytic promoters containing AP1 sites. We also found that Orf49 can activate the c-Jun and the JNK/p38 pathways. We suggest that Orf49-mediated activation of the p38/JNK pathway during lytic reactivation plays an important role in KSHV viral replication.

MATERIALS AND METHODS

Cell culture: Cos-1, 293, and Hela cells were grown in Dulbecco modified eagle medium (DMEM) supplemented with 10% FBS, penicillin and streptomycin (P/S) and 1X Gluta-max. CV-1 cells were further supplemented with Non-essential amino acids. Rhesus fibroblasts (RhF) were cultured in DMEM with 10% Cosmic Calf serum, P/S and 1X Gluta-max. The BCBL-1 cell line was grown in RPMI 1640 media with 10% FBS, P/S and L-glutamine. The BC-1 cell line was further supplemented with Sodium Pyruvate. All cell lines were maintained at 37°C and 5% CO2. BCBL-1 and BC-1 cells were induced into lytic reactivation by treating with 25ng/ml phorbol-12-tetradecanoate-13-acetate (TPA) or mock treated with the carrier dimethyl sulfoxide (DMSO). Transient transfections were carried out with the Superfect (Qiagen) reagent according to manufacturer's directions.

Plasmids: KSHV Orf49 was PCR amplified from the DNA of BCBL-1 cells induced with TPA for 48 hrs using primers 5` gcggcggaattcatggactacaaggacgacgacaa gacatcgagaaggcccc and 3` cgccgcagatctacctttttattgtatactgaacaatgc. The 5` primer added a Flag epitope to the PCR product, which was subsequently cloned into the pSG5 vector (Stratagene). The Orf50 cDNA plasmid was amplified with primers 5` cgccgcgaattcatggcgcaa-gatgacaagggtaagaagcttcggcggtcc and 3` cgccgcagatctttaacacttgtcgtcgtcgtccttgtagtcgt-ctcggaagtaattacgcc, tagged with Flag at the C-terminal end and cloned into pSG5. Orf50p, Orf57p, K8p, vIL-6p and vPolymerase promoter reporter constructs have been described previously (16, 19-22) and encode a viral promoter, which drives expression of the firefly luciferase gene in the pGL3-Basic vector (Promega). Transcription factor plasmids, also described previously (13), encode Gal-4, ATF1, ATF2, CREB or c-Jun fused to the Gal-4

DNA-binding domain. The E1B-CAT reporter plasmid contains 5 copies of the Gal-4 DNA-binding site upstream of the E1B TATA box and CAT gene.

5` and 3` Rapid amplification of cDNA ends (RACE): Total RNA from TPA induced BCBL-1 cells was reverse-transcribed using the Smart RACE cDNA amplification kit (Clonetech) with random hexamer primers. The product was used as template in a PCR reaction with one of the following Orf49 specific primers (5`-1 cttgaccaaagccgcggaacctaggt, 5`-2 ggaataagccaaattccgccctagccgc, 3`-1 tgtcgttcagatgtaccagcggtgca and 3`-2 gtccgtcgccaccctcaatccagact). 5` and 3` PCR products were cloned into pCR2-TOPO vector (Invitrogen) and individual colonies were sequenced.

RNA extractions and Northern Blots: Total cellular RNA extractions were performed with the RNA STAT-60 reagent (Tel-Test) according to manufacturer's instructions. Ten micrograms total RNA per sample were electrophoresed in 1.5% agarose-formaldehyde gels and transferred to nitrocellulose membranes (Hybond N+; Amersham Pharmacia) by capillary transfer for 16-20 hours. Radioactive probes were made by random primed oligonucleotide labeling (Random Primed DNA-labeling kit, Roche) of gel purified PCR products with [γ-^{32}P]dCTP (GE Healthcare). All hybridizations were performed using Quickhyb solution (Stratagene) for 16-20 hours at 60°C in a rotating hybridization oven. Membranes were washed twice in 2X SSC (1X SSC is 0.14M NaCl plus 0.015 soduim citrate)-0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and once with 0.1X SSC-0.1% SDS for 30 min at 60°C. Membranes were then analyzed with a PhosphorImager (Molecular Dynamics).

Western Blots: Transfected cells in 100mm dishes were washed twice with PBS harvested with 200ul radioimmunoprecipitation (RIPA) buffer (150mM NaCl, 1% NP40, 50mM Tris [pH 6.8], 0.5% sodium deoxycholate, 0.1% SDS) and freeze/thawed three times. Cell debris was spun down and the supernatant quantitated by Bradford assay (Biorad). Cell lysates were incubated with SDS gel loading buffer at 95°C for 5 min, on ice for 1 min and submitted to SDS-polyacrylamide gel electrophoresis (8). For glycosylation experiments Tunicamycin (Sigma) was added to cells to a final concentration of 20ng/ml or mock treated with an equal volume of carrier and harvested after 48 hours for Western blot analysis. Phosphatase treatments of whole cell lysates were performed following incubation with monoclonal anti–Flag antibody (Sigma) for 1 hour at room temperature and immuno-precipitation for 16 hours at 4°C with AG beads (Santa Cruz). Beads were spun down and resuspended in 100ul NEB buffer 3 and three microliters of Calf Intestinal Phosphatase (New England Biolabs) or PBS followed by incubation for one hour at 37°C. Electrophoresed gels were transferred to nitrocellulose membranes (Hybond, Amersham) in a semi-dry transfer apparatus (Biorad) at constant 20V for 1 hour and blocked in 5% dry milk, 1% Tween-20 Tris Buffered Saline (TBSt) for 1 hour at room temperature. Peroxidase-labeled antibody to Flag epitope (Sigma), total and phosphorylated c-Jun, Beta-Actin (Cell Signaling) and total and phosphorylated JNK antibodies (Upstate) were used at 1:1000 dilutions in TBSt or 5% milk-TBSt. Membranes were washed 3 times for 5 min and immunoblots detected with the SuperSignal Chemiluminescent substrate (Pierce).

Fractionation assays transfected cells were washed twice with 1X PBS and trypsinized for 5 minutes at 37°C. Trypsin was inactived by adding an equal volume of complete growth media (5% serum) and kept on ice. Cells were washed with PBS,

supernatants removed and cells resuspended in 500 microliters cytosolic lysis buffer (5mM Pipes, pH 8.0, 85mM KCl, 0.5% NP40) followed by a 5 minute incubation on ice. Nuclei were spun down and the cytosolic fraction removed. The nuclear fraction was washed with PBS, spun down and lysed with RIPA buffer. Finally, both fractions were freeze-thawed thrice and cleared by cenrtifugation.

Immuno-fluorescence Assays: Cos-1, CV-1, and RhFs were seeded in 6 well plates and transfected with 4µg DNA per well. Forty-eight hours post transfection cells were washed twice with 1X TBS and fixed in 1:1 methanol:acetone at room temperature for 1 min. Cells were washed 4 times with TBS and incubated with 1:1000 anti-Flag Cy3 (Sigma) in TBS for 1 hour at room temperature. DAPI in PBS was added for 10min followed by 2 more washes in TBS. Fluorescence was assayed in a Zeiss Axiovert 200 inverted microscope.

Promoter Reporter Assays: Six well plates seeded with CV-1 cells were co-transfected with 1.5µg promoter reporter construct and 1.5µg of pSG5, pSG5-Orf49, or pSG5-Orf50cDNA. One microgram β-galactosidase (β-gal) was co-transfected into each sample to normalize for transfection efficiency. Each sample was analyzed in duplicate in at least 2 experiments. Forty-eight hours post-transfection cells were washed once in cold 1X PBS and lysed by freeze-thawing in 1X Reporter Lysis Buffer (Promega). Lysates were analyzed for Luciferase activity with the Luciferase assay system (Promega) and for β-gal activity with the Galacto-Star Kit (Tropix) in a FLUOstar plate reader (BMG labtech).

Chloramphenicol Acetyl-Transferase Enzyme-linked Immunosorbent Assays (CAT-ELISA): CV-1 cells were seeded in 6-well plates and co-transfected with 1.2µg E1B-CAT, 1.2µg of transcription factor reporter and 1.2µg pSG5 or pSG5-Orf49. 0.6µg β-gal was

added to each sample to normalize for transfection efficiency. Forty-eight hours post-transfection cells were washed 3 times in cold 1X PBS and lysed. Cell lysates were spun down and incubated in CAT-ELISA kit (11) microwell plates according to manufacturer's recommendations. CAT expression was assayed using a FLUOstar plate reader (BMG labtech).

RT-PCR: c-Jun N-terminal Kinase (JNK) inhibitor SP600125 or p38 inhibitor SB202190 (Sigma Aldrich) was added to a final concentration of 20μM to BCBL-1 cells and simultaneously induced with TPA or mock induced with DMSO. RNA was harvested 24 hours post induction using the RNA STAT-60 reagent (Tel-Test) according to manufacturer's instructions and equal amounts were reverse transcribed with the Reverse Transcription System kit (Promega). The product was diluted ten to fifty fold and used as template for PCR reactions with primers to vGPCR, Orf57 (MTA), Orf49, LANA and cellular GAPDH.

RESULTS

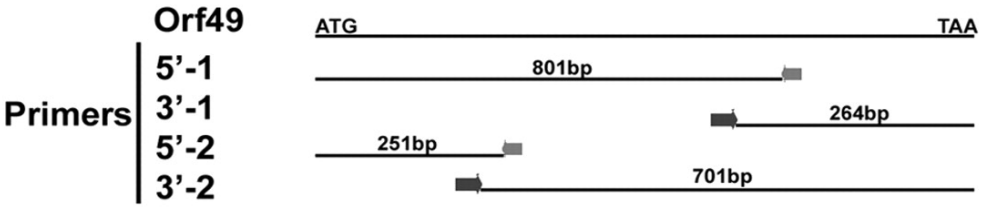
Identification of the Orf49 transcript: In an effort to identify the Orf49 transcript we performed 5' and 3' rapid amplification of cDNA ends (RACE). Total RNA was isolated from TPA-induced BCBL-1 cells and used as template for an initial reverse transcription step. Gene-specific primers were then used to amplify, clone and sequence Orf49 cDNA. We designed Orf49-specific primer sets to amplify the 5' and 3' ends of Orf49 cDNAs and produce a 200bp region of overlap between them enabling the alignment of the 5' and 3' clone sequences (Fig.1A). The PCR products (Fig.1B) were cloned into the TOPO 2.1 vector (Invitrogen). Since the length of Orf49 is about 930bp we were able to produce sequence alignments of the full-length Orf49 transcript including a short 5' untranslated region (UTR)

and the 3` poly-adenosine tail (Fig. 1C). As depicted in Fig.1C, the full-length Orf49 transcript is not spliced and the coding sequence is identical to the genomic open reading frame.

Kinetics of expression of Orf49: In order to map the expression kinetics of Orf49 we induced BCBL-1 cells with 25ng/ml TPA or vehicle (DMSO) and harvested total RNA at 12, 24, 36, 48 and 72 hours post-induction. Ten micrograms of total RNA per sample was electrophoresed on an agarose-formaldehyde gel and the resolved RNAs were transferred to nitrocellulose.

Northern blot analysis was performed with an Orf49 probe. Fig. 2 (upper panel) shows that Orf49 RNA is only transcribed in TPA-induced cells and not in the DMSO-treated cells. Orf49 was observed in two different transcripts; 3.6 and 1.2 Kb in size. The 1.2 kb transcript is very close in size to the predicted size of Orf49 while the 3.6 Kb band likely represents a poly-cistronic transcript. The Orf49 transcripts were readily detectable at 12 hrs post-induction indicating that this gene is transcribed with early kinetics. The signal peaked between 24 and 36 hrs post-induction and remained high at the 48 and 72 hour timepoints. A Northern blot with Orf50-specific probe was included (middle panel)

A



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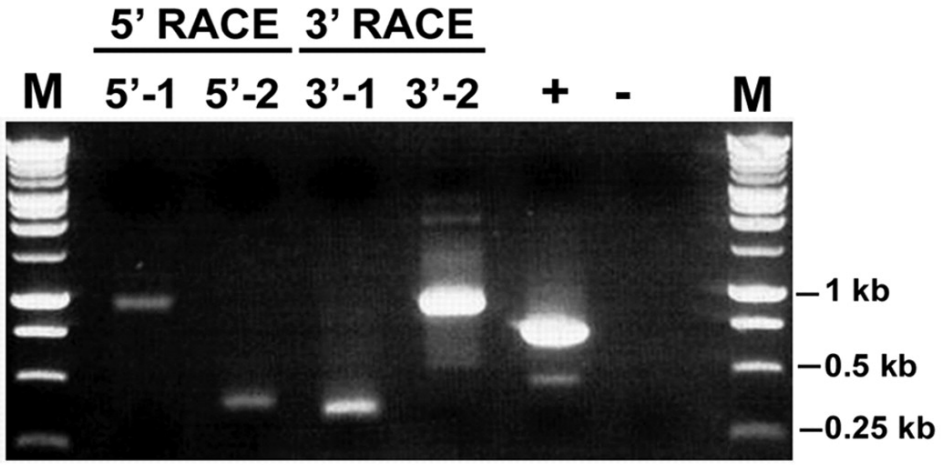

C

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Figure 1. Delineation of the full-length Orf49 transcript. A) RT-PCR design. Schematic representation of the Orf49 coding region (top) and location of RT-PCR primers designed for 5` and 3` RACE (arrowheads). **B)** Molecular cloning of RT-PCR products. Ethidium Bromide visualization of RT-PCR products. Marker band sizes are provided on the right. Lanes 5'-1, 5'-2 yielded products of ~900 and ~350 basepairs (bp) respectively. Lanes 3'-1, 3'-2 yielded products of ~340 and ~850bp respectively. Lane M: marker, Lane + : primers 5`-1 and 3`-2 yield an internal fragment of 750bp, Lane - : negative control (no reverse transcriptase).**C)** Sequence of the full-length Orf49 transcript with 5' and 3' UTRs. RT-PCR products were cloned, sequenced and aligned. A short 5' UTR and a 3` poly-A tail were identified. The transcript is not spliced and therefore identical to the genomic coding sequence.

in order to demonstrate efficient reactivation. The lower panel in Fig. 2 shows the agarose gel with equal loading of the individual RNA samples. Orf49 thus encodes a lytic protein that is transcribed with early kinetics and to high levels.

Characterization of the Orf49 protein: The cDNA for KSHV Orf49 was PCR amplified and a Flag epitope was inserted at the N-terminus of the protein. The PCR product was then cloned into the pSG5 (Stratagene) vector and transfected into Cos-1 cells. As shown in Fig. 3A, Orf49 ran as a 30kD protein on SDS-PAGE. Sequence analyses of Orf49 suggested several possible N-linked glycosylation and phosphorylation sites but no significant similarity to other known proteins or cellular localization signals. The glycosylation state of Orf49 was determined by treating cells with tunicamycin, an inhibitor of N-glycosylation, for 48 hours and performing an Orf49 Western blot. Tunicamycin treatment did not change the electrophoretic profile when compared to mock treatment suggesting that Orf49 is not N-glycosylated (data not shown).

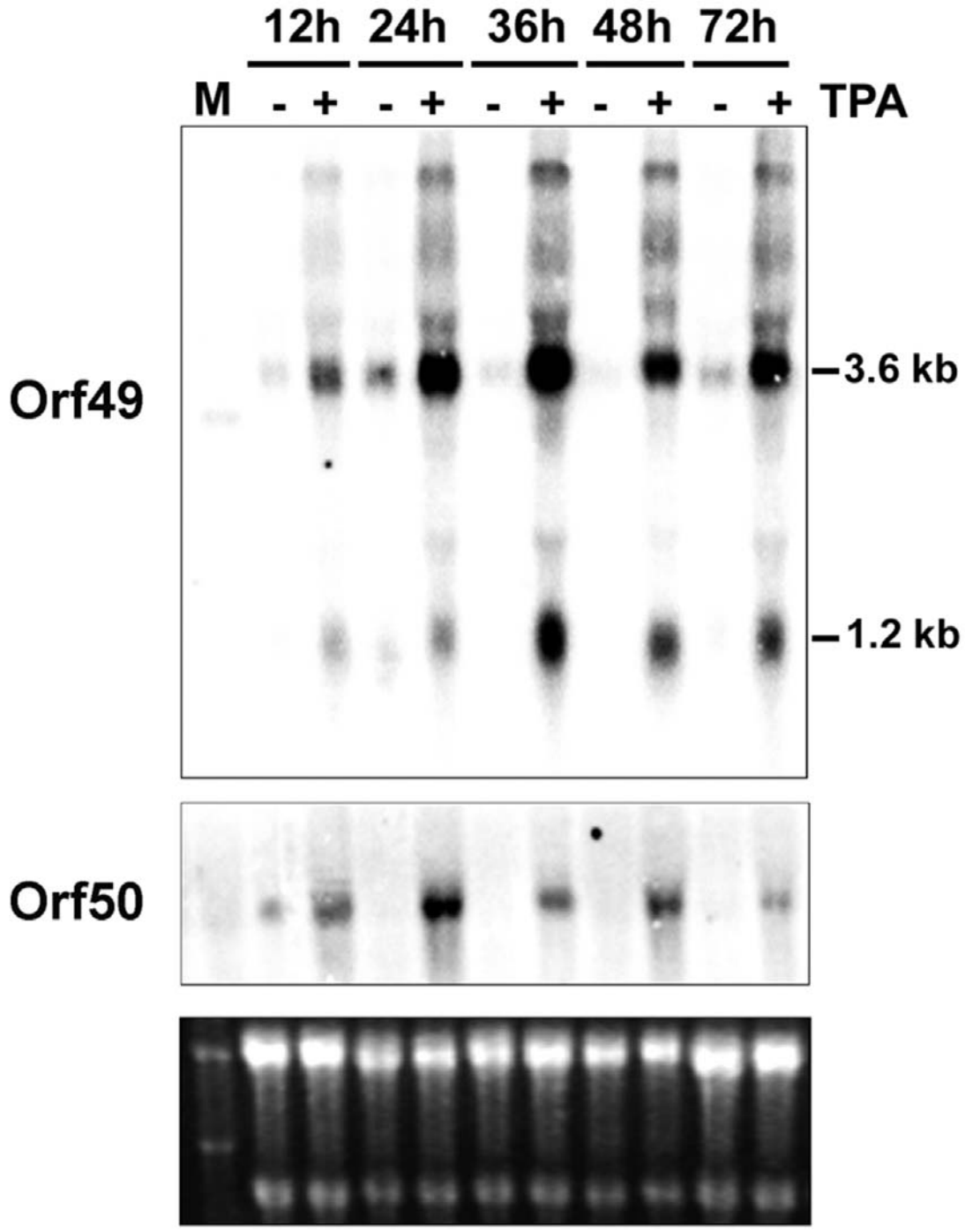
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Figure 2. Expression Kinetics of Orf49. BCBL-1 cells were induced into lytic reactivation with 25ng/ml TPA or mock treated with DMSO. Total RNA was harvested at 12, 24, 36, 48 and 72 hours post-induction and 10□g RNA loaded per well of a formaldehyde-agarose gel. Northern blot analysis with an Orf49-specific probe reveals two Orf49 transcripts (Upper panel), a longer, 3.6 Kb transcript and a shorter 1.2 Kb species. Middle panel: Northern Blot with a Orf50-specific probe to demonstrate reactivation. Lower panel: loading control as visualized by Ethidium Bromide staining of the formaldehyde-agarose gel prior to transfer onto nitrocellulose membrane.

In order to identify the phosphorylation state of the Orf49 protein we transiently transfected Cos-1 cells with Orf49-Flag plasmid and immunoprecipitated the lysates with anti-Flag monoclonal antibody. Lysates were either treated with phosphatase or mock treated with PBS followed by Western blot with anti-Flag antibody. There was no difference in migration between phosphatase treated versus mock treated cell lysates suggesting that the protein may not be phosphorylated (data not shown). However it is possible that if phosphorylated, the protein is either resistant to CIP treatment or the mobility shift is too small to be detected by conventional SDS-PAGE.

We next determined the intracellular localization of the Orf49-encoded protein. Cos-1 cells and Rhesus fibroblasts (RhFs) were transfected or mock transfected with Orf49 plasmid. Cos-1 cells were harvested using a cell fractionation method in order to separate the cytoplasmic and nuclear fractions of lysates. The nuclear, cytoplasmic and a non-fractionated, total protein sample were subjected to Western Blots with Flag antibody. As shown in Fig.3, the flag-tagged Orf49 protein is detectable in the nucleus of Cos-1 cells as well as in the cytoplasm. We used the cytoplasmic marker GRP-78 (35) as a control for the stringency of the cell fractionation method to show that there was no detectable cytoplasmic contamination in the nuclear fraction.

For immuno-fluoresence we chose RhF cells because of their elongated shape and clear demarcation of the nucleus and cytoplasm. 48 hours post-transfection RhFs were subjected to immuno-fluorescence assays using an anti-Flag antibody conjugated to Cy-3 dye, and DAPI, a nuclear stain. We found that Orf49 was present in both the cytoplasm and nucleus. Fig.4 shows immunofluorescence of Orf49 in the RhF cells. Panels a and e show bright field images of the cells, panels b and f represent staining while panels

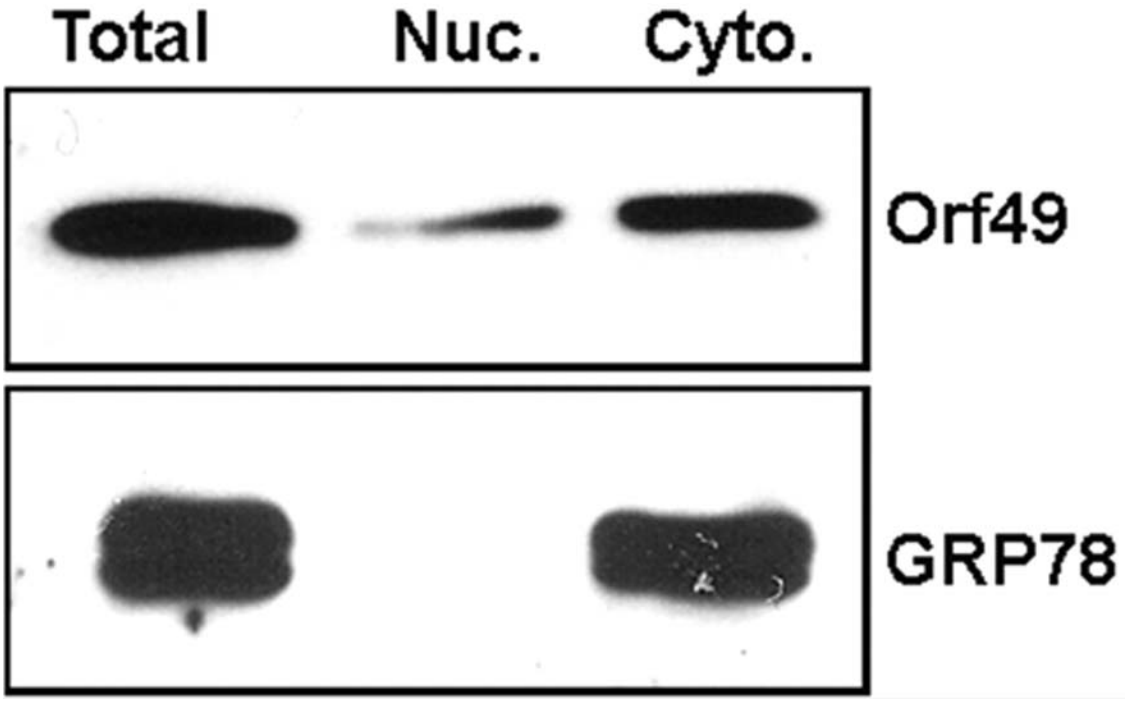
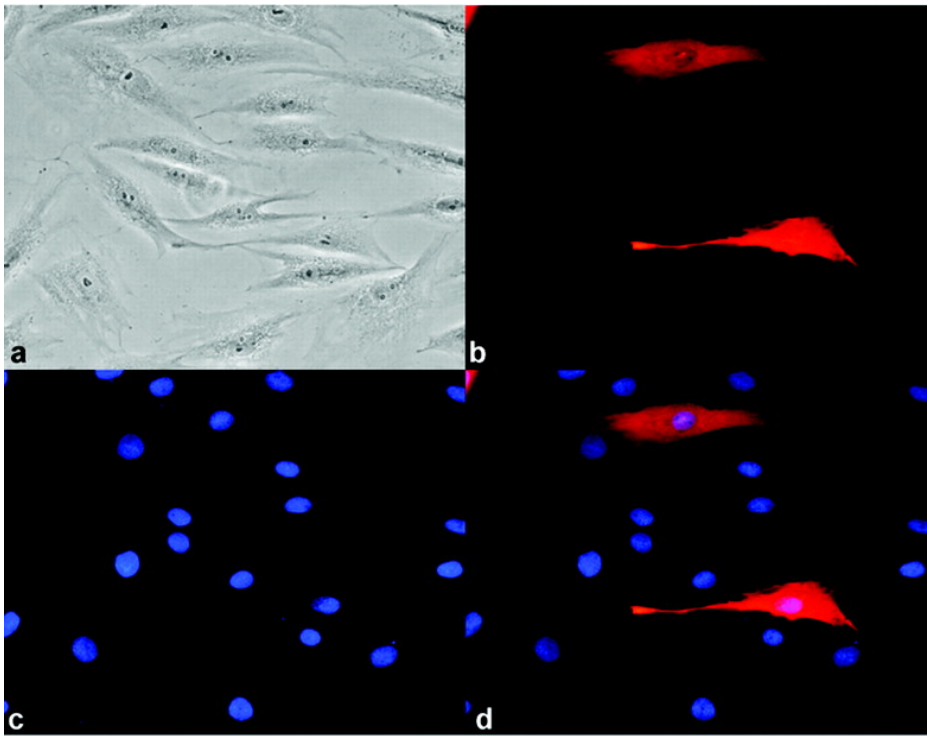


Figure 3. Cell fractionation of Orf49-transfected cells. Cos-1 cells were transfected with Orf49-Flag and separated into nuclear and cytoplasmic fractions. Equal amounts of protein were loaded onto SDS-PAGE gels, transferred to nitrocellulose and the blots were probed with Flag antibody. The upper panel shows Orf49-Flag expression in the nucleus and the cytoplasm. The lower panel shows expression of GRP-78, a bonafide cytoplasmic marker.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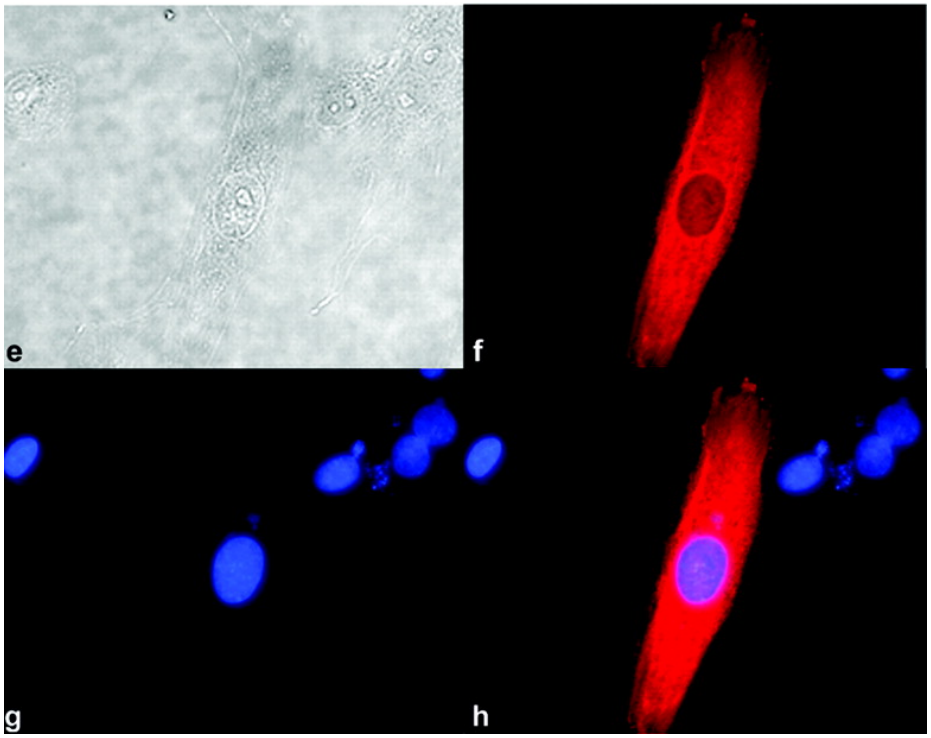
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Figure 4. Cellular localization of Orf49. Rhesus fibroblasts (RhF) were transfected with an expression vector encoding a Flag-tagged Orf49 protein. 48 hours later, cells were fixed and stained with the nuclear DAPI stain (blue) and a Cy-3-conjugated antibody to the Flag epitope (red). Orf49 is readily detected in both the nucleus and cytoplasm of the cell. Panels a and e: phase contrast, panels b and f: Cy-3, panels c and g: DAPI, panels d and h: merge DAPI and Cy-3 stains. Panels a-d at 40X magnification and panels e-h at 100X magnification.

c and g represent nuclear staining with DAPI. A merged view of Orf49 and nuclear staining is shown in panels d and h. We observed similar cellular localization profiles for Orf49 in CV-1 and 293 cells (data not shown).

Orf50-mediated activation of key lytic promoters is enhanced by Orf4: Since Orf49 was expressed with early kinetics and appeared to be both nuclear and cytoplasmic, we attempted to determine if it could activate expression from a number of KSHV lytic promoters. We co-transfected a set of promoter-luciferase reporter constructs including Orf57p, vGPCRp, and K8p with cDNA expression plasmids for either, Orf50, Orf49 or both. Although Orf49 was in itself unable to achieve high levels of activation of any of the promoters tested, we observed a cooperative effect of Orf49 with Orf50 on the activation of Orf57p, vGPCRp, and K8p (Fig. 5). Orf49 could augment Orf50-mediated transactivation of these promoters. It is important to note that Orf50-mediated induction of these promoters ranged from 50 to 300 fold over that of empty plasmid arguing for a strong enhancing effect by Orf49 over an already high level of expression. (Fig.5). There was no cooperation with Orf50 on the induction of the vIL-6, Orf50, Pan (nut-1), and viral DNA polymerase promoters by Orf49 (data not shown). Closer inspection of the promoter sequences cooperatively activated by Orf50 and Orf49 revealed that they all contain AP-1 sites, raising the possibility that Orf49 may augment Orf50 transcription in an AP1-dependent manner.

Orf49 can enhance c-Jun transcriptional function: In order to determine if Orf49 could activate transcription factors through the activation of AP-1 sites, we performed chloramphenicol acetyl-transferase (CAT) reporter assays with a c-Jun reporter plasmid. We examined whether Orf49 could augment the function of ATF-1, ATF-2, CREB or c-

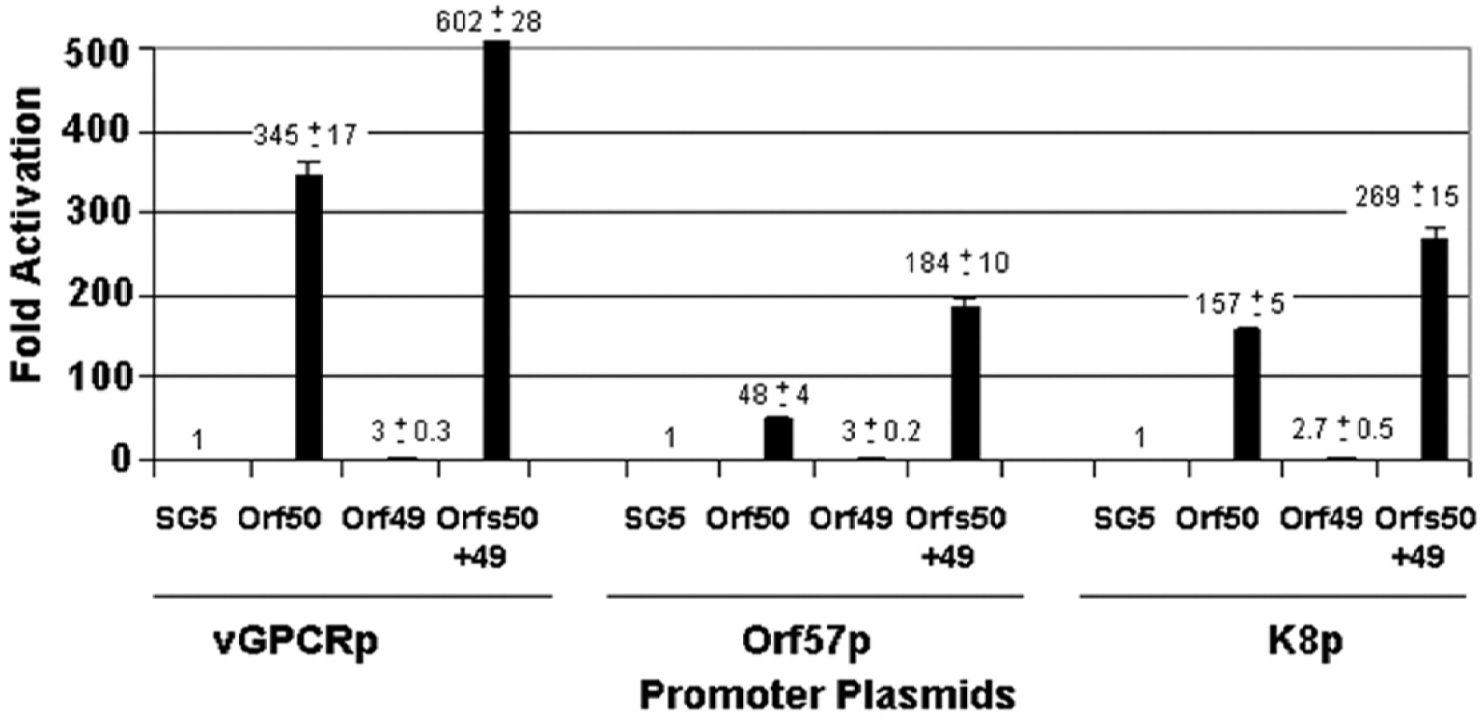

Fold Activation
500
400
300
200
100
0
602 ± 28
345 ± 17
269 ± 15
184 ± 10
157 ± 5
48 ± 4
1
3 ± 0.3
1
3 ± 0.2
1
2.7 ± 0.5
SG5
Orf50
Orf49
Orfs50 +49
SG5
Orf50
Orf49
Orfs50 +49
SG5
Orf50
Orf49
Orfs50 +49
vGPCRp
Orf57p
K8p
Promoter Plasmids

Figure 5. Orf49 enhances Orf50-mediated induction of K8, Orf57 and vGPCR lytic promoters. Promoter reporter assays: empty vector (pSG5), Orf50, Orf49 or Orf50 plus Orf49 expression constructs were co-transfected into CV-1 cells with plasmids in which the vGPCR, K8 and Orf57 lytic promoters drive luciferase expression. 48 hours post-transfection, promoter activity was determined by luciferase assay. Luciferase activities were normalized to beta-galactosidase and expressed as fold induction over empty plasmid. Fold activation values are indicated above each bar along with the standard error. Each experiment was performed in duplicate and the experiment was repeated several times.

Jun fusion proteins linked to the Gal4 DNA binding domain. Orf49 was co-transfected with ATF-1, ATF-2, CREB-1, and c-Jun Gal4 protein fusion expression plasmids into CV-1 cells and the activation of a reporter plasmid containing five copies of the Gal4 DNA binding site upstream of the CAT gene was measured by a CAT ELISA. As shown in Fig. 6, Orf49 was unable to activate transcription through the ATF1, ATF2 and CREB transcription factors. However, there was a dose-dependent, ~5 fold induction of the c-Jun transcription factor over that of empty plasmid. Since the DNA binding activity in this experiment was mediated through the Gal4 DNA binding domain of the individual fusion proteins, these results suggest that Orf49 activates promoters by enhancing c-Jun transcriptional function. Moreover, the selective activation of c-Jun but not other transcription factors could explain why Orf49 was able to enhance Orf50 induction of only those promoters with AP1 sites, given that c-Jun is one of the strongest activators of AP1 elements.

Orf49 is capable of inducing c-Jun phosphorylation: The activation of c-Jun is attributed to the phosphorylation of serine residues 63 and 73 (34, 36). In order to confirm that c-Jun was activated in cells expressing Orf49, we transfected CV-1 cells with an Orf49 expression plasmid and harvested protein lysates in the presence of phosphatase inhibitors. Western Blot analyses were performed using antibodies against total c-Jun or phosphorylated c-Jun at both serine residues 63 and 73 to determine the phosphorylation and activation state of c-Jun in the presence or absence of Orf49. Figure 7A shows that the c-Jun phosphorylation is greatly increased in the presence of Orf49 as compared to empty vector control.

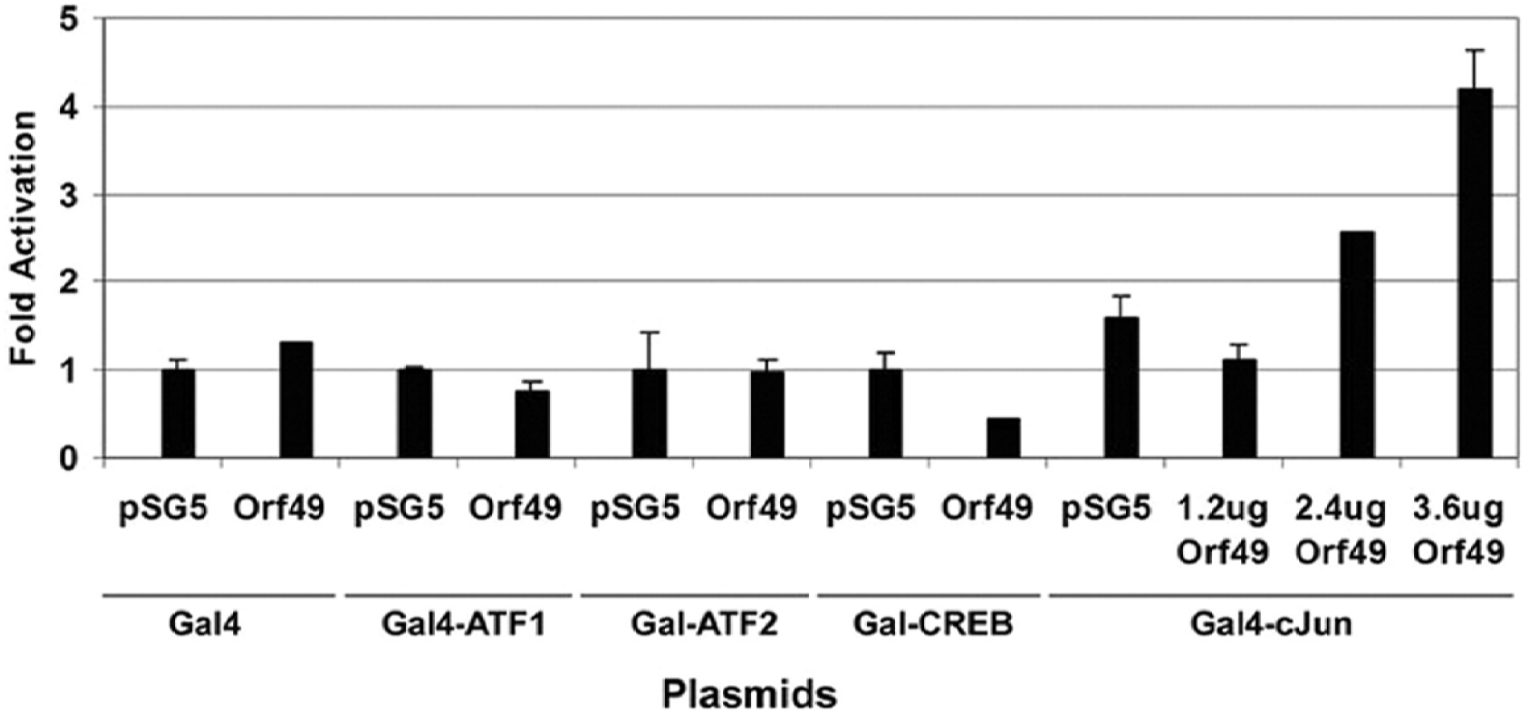

Fold Activation
5
4
3
2
1
0
pSG5
Orf49
pSG5
Orf49
pSG5
Orf49
pSG5
Orf49
pSG5
1.2ug Orf49
2.4ug Orf49
3.6ug Orf49
Gal4
Gal4-ATF1
Gal-ATF2
Gal-CREB
Gal4-cJun
Plasmids

Figure 6. Orf49 enhances c-Jun transcriptional function. pSG5 empty vector or Orf49 expression plasmids were co-transfected into CV-1 cells with plasmids encoding five copies of the Gal4 binding site upstream of the CAT gene and Gal4-protein fusions of ATF1, ATF2, CREB and c-Jun. Orf49 enhanced c-Jun-mediated transcription in a dose-dependent manner, but did not affect ATF-, ATF-2 or CREB-dependent transcription function.

c-Jun phosphorylation is mediated by c-Jun N-terminal kinase (JNK). We transfected 293 cells with an Orf49 expression plasmid and performed Western blots to determine if JNK kinase was activated in the presence of Orf49. JNK activation can be measured by the phosphorylation status of threonine 183 and tyrosine 185 residues (18). We found that Orf49 was in fact able to induce the phosphorylation of JNK in 293 cells (Fig. 7B), leading to the activation of c-Jun and c-Jun–mediated transcription. In addition, p38 kinase, another member of the mitogen-activated protein (MAP) kinase family was also phosphorylated and activated in Orf49 expressing cells as compared to the vector control (Fig. 7B).

In order to ascertain if JNK and p38 are indeed activated in the more physiologically relevant context of reactivation of latently-infected B cells, we used a BCBL-1 cell line in which the lytic switch RTA is inducible by Tetracycline (26). In these cells RTA expression is tightly regulated by Tetracyclin (or Doxycyclin) and upon induction follows the strict pattern of gene expression characteristic of herpesviruses. Western blots for phospho-JNK and phospho-p38 were performed on lysates from the RTA-TREX BCBL-1 cells and the negative control pcDNA-TREX BCBL-1 cells, with or without a 24 hour Doxycyclin induction (final concentration of 1μg/ml). Western blots for RTA were also performed to demonstrate the Dox-mediated induction of RTA. As shown in figure 7C, both JNK and p38 are activated after induction of RTA-TREX BCBL-1 cells, but not pcDNA-TREX BCBL-1 cells, thus demonstrating that RTA-induced lytic reactivation does induce phosphorylation/activation of JNK and p38 kinases and that our results are not an artifact of TPA-induction or transient transfection.

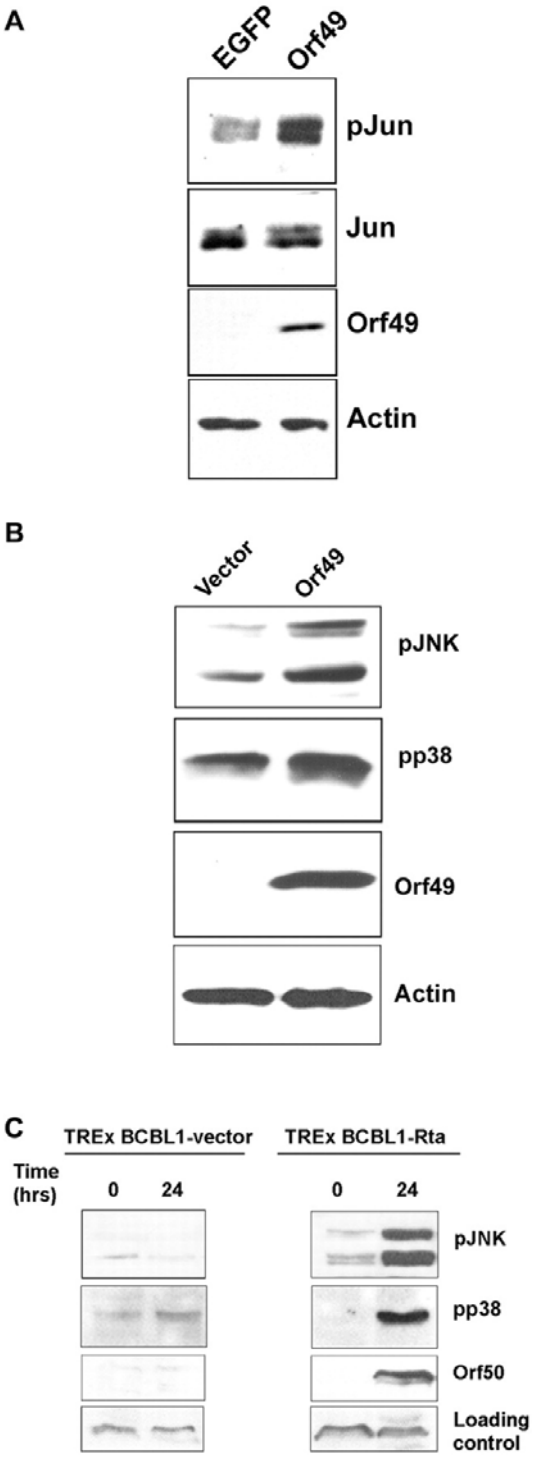
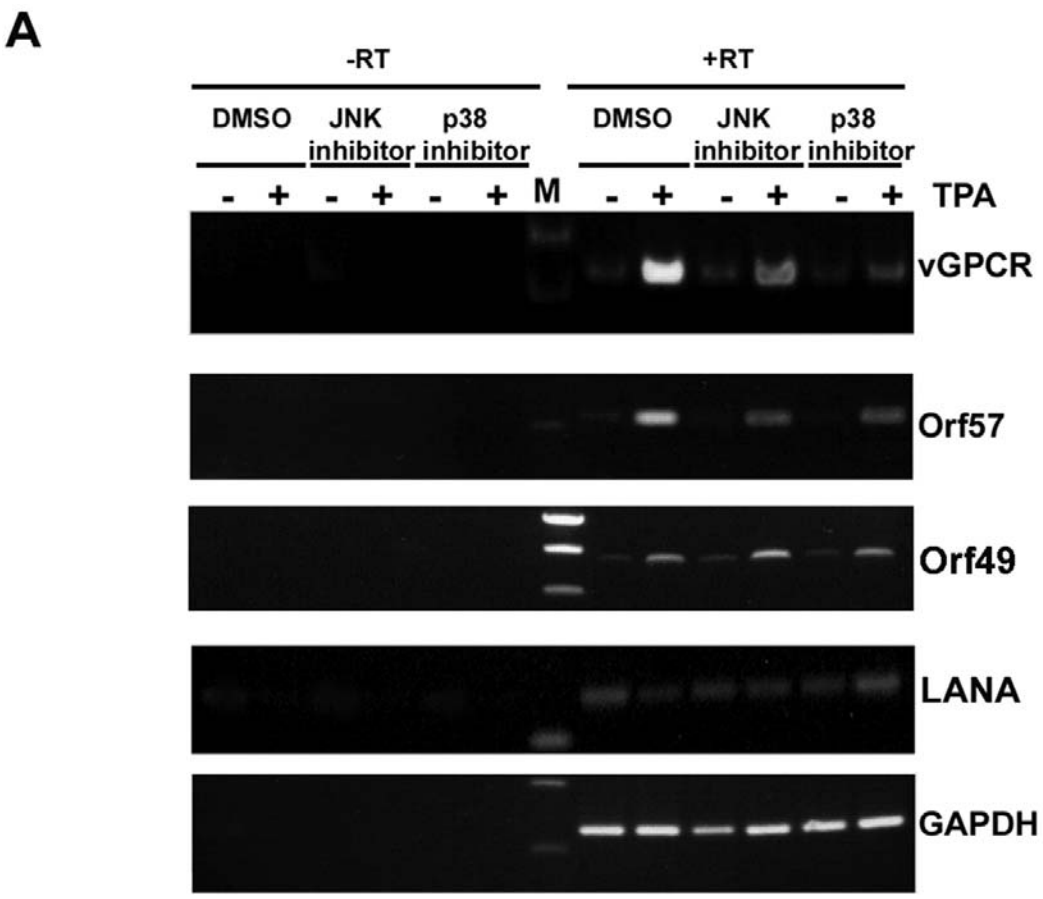

A
EGFP
Orf49
pJun
Jun
Orf49
Actin
B
Vector
Orf49
pJNK
pp38
Orf49
Actin
C
TREx BCBL1-vector
TREx BCBL1-Rta
Time (hrs)
0
24
0
24
pJNK
pp38
Orf50
Loading control

Figure 7. Orf49 induces phosphorylation of c-Jun by activation of JNK and p38.

A) CV-1 cells were transfected with EGFP vector or an Orf49 expression plasmid and cells were harvested and lysed 48 hours post transfection. Phospho-c-Jun levels increased in the presence of Orf49 (first panel) while total c-Jun levels (second panel) remained comparable. The third panel shows expression of Orf49. Actin expression is also shown in the bottom panel to indicate equivalent loading. **B)** Both JNK and p38 pathways are activated by Orf49. CV-1 cells were transfected with pSG5 vector or Orf49 expression plasmids, serum starved for 24 hours and harvested 48 hours post transfection. Lysates were subjected to Western blot analysis with anti-phospho-JNK (pJNK) and anti-phospho-p38 (pp38) antibodies. Cells expressing Orf49 (as determined by western blot with anti-Flag) showed vastly increased levels of phospho-JNK and phospho-p38 compared to the empty vector control. Actin expression is also shown in the bottom panel to indicate equivalent loading. **C)** JNK and p38 are activated during reactivation. pcDNA-TREX-BCBL-1 cells carrying pcDNA (empty) vector or RTA-TREX-BCBL-1 cells carrying a Doxycylin-inducible RTA were treated with 1 microgram/ml Doxycyclin or untreated for 24 hours. Lysates were harvested and Western blots for phospho-JNK, phospho-p38 and Orf50 were performed. Doxycyclin-induced RTA-TREX BCBL-1s showed increased levels of both phospho-JNK and phospho-p38, while pcDNA-TREX BCBL-1s cells showed no activation of JNK or p38. Orf50 expression was only induced in the RTA-TREX BCBL-1 cells as expected.


A
-RT
+RT
DMSO
JNK inhibitor
p38 inhibitor
DMSO
JNK inhibitor
p38 inhibitor
- + - + - + M - + - + - + TPA
vGPCR
Orf57
Orf49
LANA
GAPDH

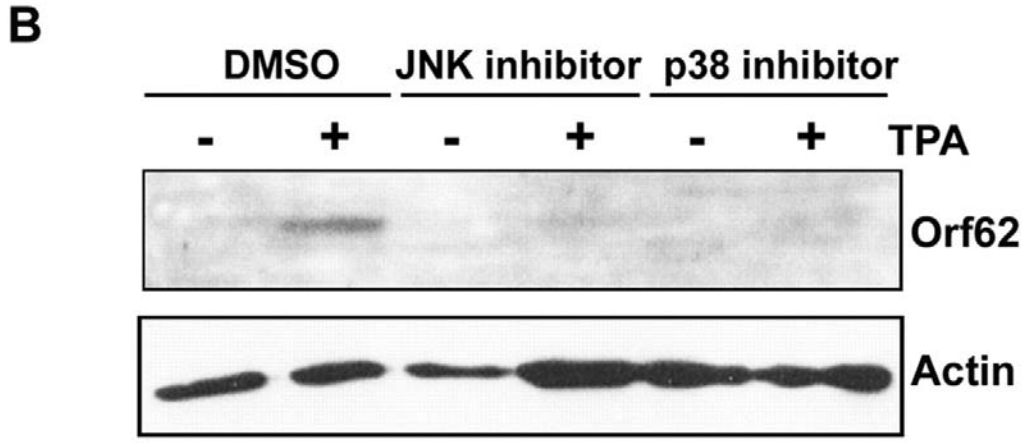

B
DMSO
JNK inhibitor
p38 inhibitor
- + - + - + TPA
Orf62
Actin

Figure 8. JNK and p38 activation is necessary for lytic gene expression. A) Reverse transcriptase-PCR (RT-PCR) of total RNAs from TPA-induced or mock-treated BCBL-1 cells treated for 24 hours with JNK inhibitor SP600125 or p38 inhibitor SB202190 was performed. PCR reactions using vGPCR and Orf57 primers revealed that expression levels of these early genes are inhibited by JNK and p38 inhibitors. However, expression of the Orf49 gene product was not inhibited by the presence of JNK or p38 inhibitors. In addition, levels of the latency gene, LANA, were not affected by the JNK or p38 inhibitors. The human GAPDH transcript was used as a loading control. B) JNK and p38 inhibition hinder late protein synthesis. BC-1 cells were induced with TPA (or mock induced with DMSO) and JNK or p38 inhibitor. Cell lysates were harvested and equal amounts of total protein analyzed by Western blots using antibody directed against the late protein, Orf62/mCP. The bottom panel shows an actin loading control.

Taken together these results define the mechanism by which the Orf49 protein activates c-Jun-mediated transcriptional activation, namely by eliciting the phosphorylation and activation of JNK/p38 MAP kinases.

JNK/p38 activation is necessary for the expression of lytic proteins: In order to determine the effect of JNK/p38 on KSHV lytic reactivation and replication, BCBL-1 cells were induced with TPA and simultaneously treated with the JNK inhibitor, SP600125 or p38 inhibitor, SB202190. Total RNA was harvested at 24 hours post-induction and reverse transcribed. The RT product was then diluted 10-50 fold and used as template in a PCR reaction with primer sets for the early lytic genes vGPCR, Orf57 and Orf49 as well as KSHV LANA and human GAPDH controls. Figure 8A shows the PCR products from these reactions. Both inhibitors seemed to decrease the levels of lytic expression of the vGPCR and Orf57 transcripts. The Orf49 transcript was unchanged in the presence of inhibitors suggesting that Orf49 expression is not refractory to JNK and p38 inhibition. The LANA and GAPDH controls were stable regardless of treatment or replicative state.

We also performed Western blots to examine the expression of late lytic proteins. Cell lysates were harvested 24 hours after treatment and equal amounts of protein were analyzed by Western blot with anti-KSHV Orf62 antibody. Orf62 is also called minor capsid protein (mCP) or TRI-1(27). As shown in Figure 8B, treatment with both JNK and p38 inhibitors suppressed lytic reactivation as measured by expression of Orf62 protein. In summary, these results support a functional role for the JNK and p38 pathways in lytic reactivation.

DISCUSSION

We report here the first characterization of KSHV Orf49 located in between the two exons coding for Orf50 and on the opposite strand. We performed 5' and 3' RACE to identify ends of the Orf49 transcript and found it to be unspliced. The Orf49 transcript was ~1000 base pairs long, containing the expected coding sequence of over 900 bases plus a 15 nt 5` UTR and a 67 nt 3' UTR including the poly-A tail. Northern blot analyses revealed that Orf49 transcripts were highly expressed during lytic reactivation with early kinetics and throughout the length of TPA treatment. Two transcripts were detected with a Orf49-specific probe, one of 1.2Kb and a second of 3.6Kb. The 1.2Kb transcript corresponds closely to our full-length cDNA clone. However, it is possible that there are longer, possibly bi-cistronic, transcripts that we did not detect via RACE. Expression levels peaked at 36 hours post TPA induction by Northern blot analyses. These results are similar to those reported by others (29) and personal communication with Dr. Dirk Dittmer who found that Orf49 expression peaks between 24 and 48 hours using microarray analyses.

The cDNA encoded by Orf49 expressed a protein of ~30 kilodaltons in size. We determined that the Orf49-encoded protein is not N-glycosylated nor phosphorylated despite the presence of several possible glycosylation and phosphorylation sites in the coding sequence. However it is possible that the protein can be phosphorylated but is resistant to calf intestinal phosphatase. Additionally, the Orf49 protein was localized to both the nucleus and the cytoplasm of cells as observed by immunofluoresence and cell fractionation Western blots.

We next assessed the role of Orf49 in viral transcription. We used promoter reporter assays to evaluate if Orf49 was in fact able to induce expression from lytic viral promoters.

Our results showed that Orf49 itself was not a potent transactivator but was able to cooperate with Orf50 to increase Orf50-mediated activation of the K8, Orf57 and vGPCR promoters. It is important to note that the activation of these promoters by Orf50 alone was already high ranging from 50-fold Orf57 to 350-fold (vGPCR) over that of empty vector. This strong activation of Orf57, vGPCR and K8 suggest that Orf49 synergizes with Orf50 to increase the efficiency of lytic reactivation. Indeed, Orf57 has been shown to bind Orf50 and induce expression from the Orf50 promoter (23) suggesting that, in vivo, induction of Orf57 could indirectly enhance Orf50 expression and thus lytic reactivation.

The K8 protein binds the oriLyt and is essential for lytic replication (1), hence, the increase in K-bZip expression mediated by Orf49 could significantly enhance the efficiency of lytic reactivation initiated by Orf50. Finally, the constitutively activated viral G protein-coupled receptor, vGPCR, is an early lytic gene that plays important roles in angiogenesis. Thus, cooperative induction of these three viral promoters by Orf49 and Orf50 has tangible biological consequences for host cells undergoing lytic replication as well as the surrounding environment.

We have also identified a mechanism by which Orf49 induces expression of K8, Orf57 and vGPCR. These promoters all share AP-1 sites in addition to RTA-responsive elements (RREs). Using CAT-reporter assays we demonstrated that Orf49 can augment c-Jun-mediated transcriptional activation and that this activation was dose-dependent. These results were further validated by assessing phospho-c-Jun levels in the presence or absence of Orf49. Expression of Orf49 increased phospho-c-Jun levels as compared to the vector controls. Orf50 has been shown to increase the levels of total c-Jun in DG75 cells, however it did not induce c-Jun phosphorylation (40). Together these findings could suggest a

mechanism by which Orf50 increases total c-Jun protein, and Orf49 induces phosphorylation and activation of c-Jun thus enhancing the total c-Jun transcriptional activation potential in the cell.

Since c-Jun activation is dependent on c-Jun N-terminal Kinase (JNK), we also performed anti-phospho-c-Jun N-terminal kinase (JNK) Western blots and found that Orf49 can indeed activate JNK. These results could indicate that c-Jun-mediated transcriptional activation is induced by Orf49 through JNK activation. Interestingly, Orf49 also induced phosphorylation/activation of the p38 kinase. Both JNK and p38 pathways are activated by stress, growth factors and cytokines. They are activated by the same mitogen activated protein kinase kinase kinase (MAP3Ks), share the MAP2K (mitogen activated protein kinase kinase) MKK4, and are often co-activated (43). Signaling through both pathways can result in proliferation, differentiation or apoptosis although p38 activation is also involved in development, inflammation and stress responses.

KSHV vGPCR has also been shown to trigger the activation of JNK and p38 (2) underscoring the significance of these pathways during lytic replication. Because of the wide-ranging effects of these pathways we wanted to assess the contribution of each pathway to lytic reactivation. JNK and p38 inhibitors blocked TPA-induced expression of early genes like vGPCR and Orf57 as well as late genes like Orf62/mCP. There was a marked decrease in the total amount of Orf62/mCP detected in both JNK and p38 inhibitor–treated, lytically reactivated cell lysates indicating that signaling through both pathways can influence, at least, the amount of late structural proteins produced.

KSHV Orf49 is the positional homologue of the EBV BRRF1 gene encoding the Na protein. Na has been shown to encode a 34 kD protein with early kinetics that is induced by ZTA (34). Hong et al. reported that EBV Na functions in the reactivation of latent EBV, by inducing lytic promoters and increasing the amount of viral progeny released (13). They further showed that the BRRF1 promoter was induced by ZTA through a CRE motif (ZII) and that Na enhanced EBV RTA's ability to induce ZTA, thus creating a self-reinforcing loop. EBV Na was also shown to activate c-Jun-mediated transcription. These findings led to the identification of Na as a transcription factor that activates ZTA expression through the activation of c-Jun and cooperates with RTA (BRLF1) in reactivation of EBV from latency. However, since KSHV K8, the EBV ZTA homolog, cannot by itself reactivate the virus from latency, it seems likely that the main function of KSHV Orf49 is to augment Orf50 transactivation of a sub-set of early promoters.

Thus, Orf49 represents an important member in the lytic reactivation cascade that enhances transcription from key Orf50-responsive promoters through the transcriptional activation of c-Jun and also activates the JNK/p38 kinase pathways, which are necessary for KSHV viral reactivation from latency as well as viral replication.

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CHAPTER THREE

KSHV ENCODES A VIRAL DEUBIQUITINASE

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(Manuscript in revision in PLoS Pathogens)

ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV) is etiologically linked to Kaposi's sarcoma, primary effusion lymphomas and multicentric Castleman's disease. Like other herpesviruses, KSHV can exist in either a lytic or a latent phase during its lifecycle. We report that the lytic protein encoded by KSHV open-reading frame 64 (Orf64) is a viral deubiquitinase (DUB) enzyme capable of deubiquitinating cellular proteins *in vitro* and *in vivo*. Orf64 deubiquitinase activity is effective against lysine 48 (K48) and lysine 63 (K63) linked ubiquitin chains. Orf64-encoded DUB activity lies within the first 205 residues of the protein and deubiquitination is dependent on a cysteine at position 29, since mutation of this residue ablated this activity. Cell fractionation studies revealed that the N-terminus and the full-length protein localized to both the nuclear and cytoplasmic compartments. Promoter reporter assays showed that Orf64 could synergize with the lytic switch protein, RTA, to enhance transcription from RTA-dependent lytic promoters. The function of Orf64 was tested by siRNA knockdown studies on latently infected cells that were induced into lytic replication. These experiments indicated that Orf64 plays a role in lytic replication since transcription and expression of lytic proteins was decreased in cells with reduced Orf64 levels. In sum, KSHV Orf64 encodes a robust deubiquitinase, which is unique in at least two respects. First, it does not show specificity towards K48 or K63 ubiquitin linkages. Second, Orf64 appears to enhance RTA-mediated transactivation of KSHV lytic genes. Altogether, these characteristics suggest a novel role for Orf64, a tegument protein, in the KSHV lifecycle.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV) also known as human herpesvirus 8 (HHV-8) is the most recently discovered human herpesvirus and the only human rhadinovirus [1]. KSHV is the etiological agent of Kaposi's sarcoma (KS), as well as the lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) [2,3,4]. KSHV is consistently found in all clinical forms of KS, PEL, and MCD. Moreover, KS is the most common neoplasm in the HIV-positive population and is an AIDS-defining illness [5]. Although KS lesions most frequently develop on the external skin, they can also be found on internal organs and in the oral cavity. KS is a highly angiogenic complex lesion that is comprised of endothelial cell-derived spindle-shaped cells and inflammatory cells that have migrated to the lesion [6].

Like all herpesviruses, KSHV exhibits two distinct phases in its lifecycle: a lytic phase and a latent phase. *De novo* infection of endothelial cells results in temporary lytic replication [7,8]. Lytic replication is characterized by the transcription of the entire complement of viral genes (immediate early, early, and late) in a temporal fashion. Viral replication and virion assembly is followed by the release of infectious progeny from the infected cell. In contrast, during latency, only a few viral proteins are expressed and the viral genome remains in an episomal state. Neither mode of existence is in itself capable of sustaining neoplastic growth, as each state seems to make distinct contributions that are necessary for the development of KSHV-associated malignancies [9]. Thus, lytic and latent proteins modulate the environment of the host cell to the advantage of the virus. The role of ubiquitination in KSHV replication, transformation or pathogenesis has been examined to some extent. During the lytic cycle, KSHV expresses three E3-like ubiquitin ligases

[10,11,12] encoded by open reading frames (Orfs) K3 (MIR 1), K5 (MIR2) and RTA (Orf50). Both K3 and K5 target MHC class I molecules for degradation in order to hinder presentation of viral antigens. The lytic switch protein, RTA, targets IRF-7 for degradation in order to preclude induction of the interferon-mediated antiviral state [12].

Ubiquitin (Ub) is a 76-amino acid molecule that is widely conserved and can be post-translationally conjugated to specific target proteins through different linkages. The host cell utilizes Ub to control cellular processes such as protein expression, apoptosis, cell cycle regulation, receptor trafficking, DNA repair, and signal transduction [13,14]. Moreover, antigen presentation of pathogen-derived proteins requires ubiquitin-mediated proteosomal processing [15]. The ubiquitin pathway consists of a single E1 protein that activates and transfers Ub to one of several conjugating enzymes known as the E2 adaptor proteins in an ATP-dependent fashion. The E2 protein subsequently engages an E3 ligase bound to a specific target protein. The Ub moiety is then covalently linked to the target protein through a lysine residue by the E3 ubiquitin ligases [16].

Proteolysis, however, is not the only outcome of ubiquitination. Typically, poly-ubiquitination through the lysine 48 residue of ubiquitin results in the degradation of the target protein, whereas mono- and di-ubiquitination through lysine 63 is associated with cellular transport or functional modulation of the target protein (reviewed in [17]). Poly-ubiquitination has also been demonstrated to occur through other lysine residues of ubiquitin such as lysine 29, although these linkages do not appear to be frequently used [18]. In addition, mono-ubiquitination has been shown to be involved in endocytosis, histone regulation and retrovirus budding (reviewed in [19]). Thus, ubiquitin modification represents a regulatory mechanism for multiple functions in the cell.

Besides KSHV, other herpesviruses such as HSV-1 and MHV-68 also encode E3 ubiquitin ligases [20,21]. In a parallel, but opposing fashion, herpesviruses have also pirated deubiquitinase enzymes. Deubiquitinase (DUB) proteins cleave Ub molecules from either the E3 ligase, or the target protein, thereby pre-empting ubiquitin-mediated regulation. DUBs have been identified in several herpesviruses [22]. The multifunctional, large tegument protein encoded by UL36 in Herpes Simplex virus-1 (HSV-1) and Pseudorabies virus (PRV) (α-subfamily) are DUBs, as is the homologous UL48 protein in human Cytomegalovirus (β-subfamily) [23].

Although the targets and direct effects of the herpesviral deubiquitinases are not known, studies of HSV-1 mutants indicate that the HSV UL36 plays a role in virion transport through microtubules [24], release of the viral genome into the nucleus [25,26], tegumentation and envelopment [27] as well as virion egress [28]. DUB activity of HSV UL36 appears to be important as a DUB-null mutant displayed a 3-log decrease in titers and a 50% reduction of egressing capsids from neurons [29]. Null mutations for PRV UL36 are lethal *in vitro* [30] and delay neuroinvasion in the mouse model [31]. Moreover, specifically mutating the DUB catalytic domain reduces titer and plaque size [32]. Recently, it was shown that a DUB mutant of Marek's disease virus was severely impaired in its ability to induce lymphomagenesis [33].

We report the first description of a viral DUB encoded by KSHV Orf64. Not much is known about KSHV Orf64 except that it has recently been identified as a lytic protein that is present in the tegument of the virion, and appears to act as a scaffold protein during tegumentation [34]. We found that KSHV Orf64 encoded a powerful deubiquitinase activity as measured by *in vitro* deubiquitination assays. Additionally, KSHV Orf64 was also able to

deubiquitinate cellular proteins when expressed in various cell types. The DUB activity was limited to the N-terminal domain of KSHV Orf64 and unlike other herpesviral DUBs, which show specificity for K48 ubiquitin linkages [23,35], KSHV Orf64 appears to deubiquitinate both K48- and K63-linked ubiquitin chains. KSHV Orf64 also appeared to modulate RTA-mediated transactivation of RTA-responsive promoters in reporter assays. We found that siRNA knockdown of KSHV Orf64 resulted in decreased reactivation from latency and decreased viral replication.

MATERIALS AND METHODS

Cell Culture: HeLa, CV-1 and HEK-293 cell lines were maintained in complete media: Dubelcco's Modified Eagle Media (DMEM) (Cellgro) with 10% fetal bovine serum (FBS), L-glutamine, and penicillin plus streptomycin. The rKSHV.219-Vero [41] (a kind gift from Dr. Jeff Vieira) were maintained in complete media with 5μg/ml puromycin. KSHV-293 cells were established by overlaying supernatants from reactivated rKSHV.219-Vero on naive 293 cells followed by puromycin selection at 1μg/ml. All cells were grown in a 37°C incubator, with 5% CO_2.

Plasmids: The N-terminus of Orf64 ($Orf64_N$) was constructed by PCR amplification of the first 615 nucleotides of Orf64 with a C-terminal Flag epitope and cloned into the EcoRI and HindIII sites of the pcDEF3 vector with forward primer "cgccgcgaattcgacatggcagcccagcctct-gtacatggaggg" and reverse primer "cgccgcaagctttcacttatcgtcgtcatccttgtagtcgtagtcatgtgggata-aagtaaaggaagc". The $Orf64_N$ fragment was also sub-cloned into pGEX6p-1 (Amersham) to construct a GST fusion of $Orf64_N$ (GST-$Orf64_N$). Site-directed mutagenesis of $Orf64_N$ was conducted to change the cysteine residue at amino acid 29 to a glycine residue (C29G) generating the plasmid, $Orf64_{N\text{-}C29G}$ and GST-$Orf64_{N\text{-}C29G}$. The full-length Orf64 ($Orf64_{FL}$)

was PCR amplified and cloned into the pcDEF3 vector with a C-terminal Flag epitope using the enzymes EcoRI and Not I with primers "cgccgcgaattcgacatggcagcccagcctctgtacatggaggg" and "cgcgcggcggccgctcacttatcgtcgtcatccttgtagtccaagtaccact-tcttttaactgtcaacgc", respectively. PCR products were digested with the appropriate enzymes and ligated into pcDEF3. Five microliters of the ligation reaction was transformed into Stbl2 (Invitrogen) bacteria. The bacteria were grown at 30°C overnight on LB plates with 50μg/ml ampicillin. Clones were screened by enzymatic digestion and sequencing.

Luciferase Assay: Orf50, Pan (nut-1), glycoprotein B (gB), vGPCR and DNA-Binding protein (DBP) promoter-reporter constructs have been previously described [47,48,49]. $Orf64_{FL}$ or empty expression vector was cotransfected with β-Gal, the indicated promoter-reporter constructs, and an RTA expression plasmid into HEK-293 cells. Forty-eight hours post-transfection, cells were washed in cold PBS and lysed with Reporter Lysis Buffer (Promega) for 5 minutes at room temperature. Cells were frozen and thawed once and centrifuged at 3500 rpm for 5 minutes. Samples were analyzed for luciferase activity with the luciferase assay system (Promega) and for β-Galactosidase with the Galacto-Star kit (Tropix). Luciferase activity was normalized to β-Gal and expressed as fold induction over empty vector. Luciferase assays were performed in triplicate and each experiment was repeated at least four times.

GST-$Orf64_N$ protein purification: BL21 (DE3) cells were transformed with plasmids pGEX-6p-1 (control vector), pGEX-6p-$Orf64_N$ or pGEX-6p-$Orf64_{N\text{-}C29G.}$ The bacteria were then grown in 2XYT broth with 50μg/ml ampicillin and 34μg/ml chloramphenicol to an OD_{600} of 0.6 as per the manufacturer's directions and induced with 1mM IPTG for two hours at room temperature. Cells were spun down, and resuspended in lysis buffer (200mM NaCl,

50mM Tris-HCl, 0.5mM EDTA, 5% Glycerol, 0.5% NP40 plus Complete protease inhibitor cocktail [Roche]) followed by sonication. Triton-X was added to the lysates to a final concentration of 1% and the samples were rocked for 30 minutes followed by centrifugation at 13,000 rpm for 10 minutes. Supernatants were transferred to new tubes and incubated with a 50% slurry of glutathione-sepharose beads in 1X PBS for one hour. The GST-tagged proteins were washed three times with 1X PBS and used for *in vitro* deubiquitination assays.

In vitro deubiquitination (DUB) Assays: *In vitro* DUB assays were performed in DUB assay buffer containing 50mM Hepes, 0.01% Brij-35, and 3mM DTT [50]. Synthetic Lysine 48 (K48) or Lysine 63 (K63) ubiquitin chains (Biomol) were added to purified GST-Orf64$_N$ purified protein or 40ng of purified Isopeptidase T (Biomol) (positive control), at 37°C for 3 to 4 hours. The sample was subsequently loaded onto a 10% SDS denaturing gel, electrophoresed and transferred to nitrocellulose. Western blots were performed with an anti-ubiquitin antibody (Sigma). For the *in vitro* fluorescence DUB assays, the Ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC) substrate (Biomol) was added to GST-Orf64$_N$ purified protein in AMC Buffer containing 50mM Hepes, 0.5mM EDTA, 1mM DTT and 0.1mg/ml BSA. The samples were then pre-treated or mock-treated with the suicide inhibitor ubiquitin aldehyde (Ub-Al) (Santa Cruz). After incubation at room temperature at the indicated times, fluorescence (excitation at 380nm, emission at 460nm) was measured using the Fluostar machine (BMG).

In vivo deubiquitination assay: Deubiquitination assays were also performed in transfected cells. HeLa, HEK-293, CV-1 and rKSHV.219-Vero cells were transfected with 2µg WT-Ub, K48-Ub or K63-Ub expression plasmids (kind gift of Dr. Vishva Dixit [39]) and 8µg pcDEF3-Orf64$_N$, pcDEF3-Orf64$_{FL}$ or empty vector pcDEF3. Forty-eight hours post-

transfection, cells were lysed with NP40 lysis buffer and equal amounts of protein lysate was loaded into 10% SDS-PAGE gels for Western blot analysis with anti-HA-HRP antibody.

Cell fractionation assay: 293 cells in 100mm plates were transfected with pcDEF3-Orf64$_N$ or empty vector, pcDEF3. Cells were harvested 48 hours later by trypsinization for 5 minutes at 37°C. Complete media was added to neutralize trypsin, followed by washing twice with cold PBS. The plasma membrane was disrupted with 500µl cytosolic lysis buffer [5mM PIPES, 85mM KCl and 0.5% NP40 with complete (Roche) protease inhibitors] and incubated on ice for 5 minutes. Nuclei were spun down and the cytosolic fraction was transferred to a fresh tube. The nuclei were washed three times with 1X PBS, then lysed in 250µl radioimmunoprecipitation (RIPA) buffer (150mM NaCl, 1% NP40, 50mM Tris [pH 6.8], 0.5% sodium deoxycholate, 0.1% SDS with complete protease inhibitors (Roche)) and freeze/thawed three times. Cell debris was spun down and the supernatants quantitated by Bradford assay (Biorad).

Western blot analysis: Cells in 100mm dishes were transfected using Superfect reagent (Qiagen). Cells were harvested 48 hours post-transfection, washed twice with PBS, and lysed with 400µl Nonidet P-40 buffer (150mM NaCl, 1% NP40, 50mM Tris [pH 6.8], with complete protease inhibitors (Roche)). Cell debris was spun down and the supernatant quantitated by Bradford assay (Biorad). Cell lysates were incubated with SDS gel loading buffer at 65°C for 5 minutes, and subjected to SDS-polyacrylamide gel electrophoresis. Electrophoresed gels were transferred to nitrocellulose membranes (Hybond, Amersham) by wet transfer (20V for 16 hours) and blocked in 5% non-fat dry milk (NFDM), 1% Tween-20 Tris Buffered Saline (TBSt) for 30 minutes at room temperature. Flag-HRP antibody (Bethyl) was used at 1:10,000 dilution in 3% NFDM-TBSt. HA-HRP antibody (Sigma) was

used at 1:10,000 dilution in 1% BSA in TBS-T. Goat anti-actin antibody (Cell Signaling) was used as a control at 1:1,000 dilution and the secondary peroxidase-conjugated anti-goat antibody (Dako) was diluted to 1:2,000, both in 5% NFDM. Rabbit anti-ubiquitin (Sigma) was used at 1:250 and secondary anti-rabbit peroxidase antibody (Amersham) was diluted to 1:10,000, both in 5% NFDM. GRP-78 antibody (Santa Cruz) was used at 1:500 dilution in 5% NFDM. Anti-tubulin (Sigma) was diluted to 1:10,000 and the anti-mouse peroxidase-conjugated secondary antibody (Cell Signaling) was used at 1:2,000, both in NFDM. Membranes were washed for 30 minutes with TBST and Western blots were incubated in SuperSignal Chemiluminescent substrate (Pierce).

Viral reactivation and replication assay: KSHV-293 cells in 100mm plates were either mock induced with 1:1 serum-free DMEM:SF9 medium or induced with 1:1 serum-free DMEM:Bac50 in SF9 medium for one hour at 37°C. The inoculum was removed and replaced with complete medium in the mock-induced set, or complete medium with 1.7mM Sodium Butyrate in the induced set. Twenty-four hours post-induction, cells were transfected with siRNAs against luciferase (Luc) or siRNAs against Orf64 in duplicate. Both sets were incubated for 48 hours post-transfection, at which point the supernatants and cells were harvested. The cell sample was split into two fractions to isolate both RNA and protein. Total cell RNA extractions were performed with the RNeasy Plus Kit (Qiagen) according to manufacturer's instructions followed by RNase-free DNase treatment at 37°C for 1 hour. An additional step to remove enzyme and digested DNA was performed with the RNeasy miniprep kit (Qiagen). One microgram total RNA was used in a reverse-transcription reaction (Promega). Each reaction volume was brought up to 120µl and used as template in

PCR reactions. For cell extracts, cells were lysed with NP40 lysis buffer as described above and Western blots were performed as described above with anti-vIL6 antibody (ABI).

RESULTS

Clustal alignment of KSHV Orf64 homologs in other herpesviruses. HSV-1 UL36 and EBV Orf BPLF-1 were aligned with KSHV Orf64 using the Clustal W sequence alignment tool. Figure 1 shows the first 500 amino acid (aa) residues of these homologs, which display a low degree of identity and similarity. Notably, several short, well-conserved motifs emerge. The cysteine, histidine, and aspartic acid residues in HSV-1 (amino acid positions 66, 197 and 199) and KSHV Orf64 (amino acid positions 29, 159, and 161) are conserved. These three amino acids represent a catalytic triad that constitutes the catalytic core for deubiquitinase activity [36]. These residues are conserved in other human herpesvirus homologs as well [35], thus providing strong indication that these residues constitute the catalytic core of the DUB.

Orf64 protein expression and intracellular localization. The full–length Orf64 ($Orf64_{FL}$) and N-terminus fragment of Orf64 ($Orf64_{N}$) encoding the first 205 amino acids were each tagged with the Flag epitope and cloned into the pcDEF3 [37] mammalian expression vector. In order to determine the molecular size of these proteins, the individual plasmids were transfected into HEK-293 cells. Forty-eight hours post-

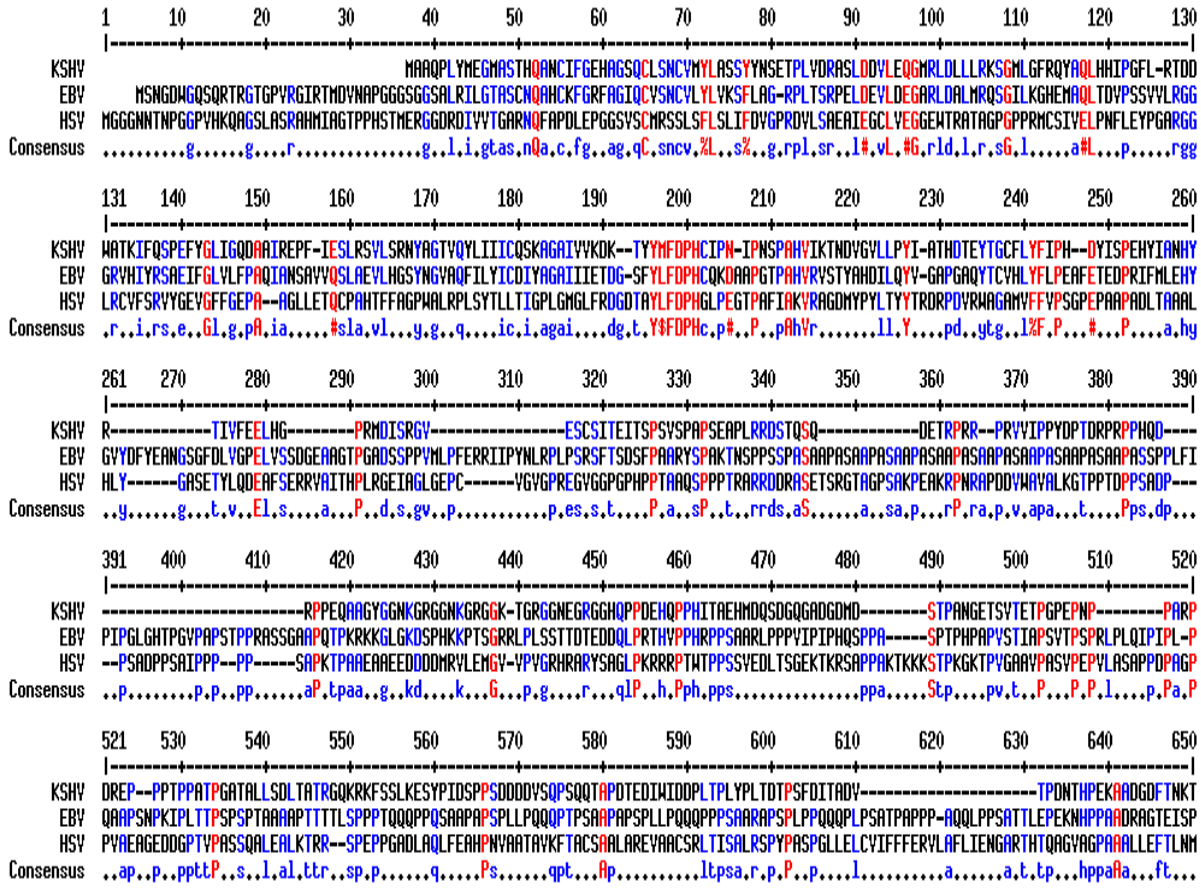
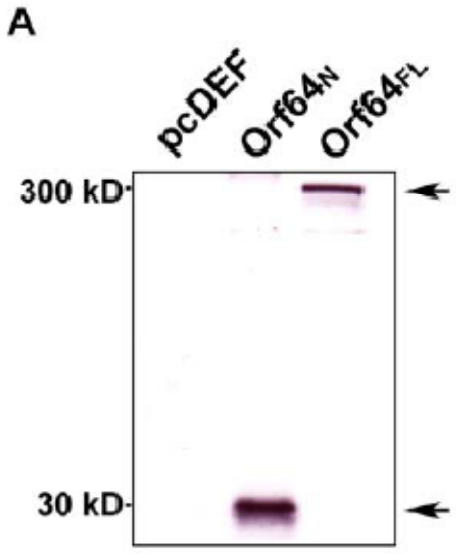


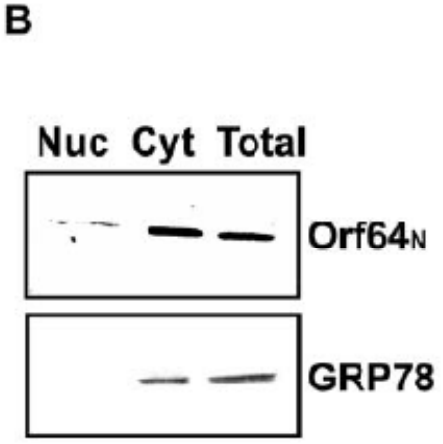
Figure 1. Sequence analysis predicts a deubiquitinase in KSHV Orf64. Clustal W alignment of the amino terminal 500 amino acid residues of KSHV Orf64 and homologs from EBV (Orf BPLF1) and HSV-1 (UL36) reveal key conserved amino acids shown in red. Red arrows indicate components of the putative catalytic core of the cysteine protease.

transfection, cells were harvested and lysed, and subjected to Western blot analysis using a Flag-HRP antibody. As depicted in Fig. 2A, the N-terminus of Orf64 ran at a mobility of 30kDa while the full-length Orf64 ($Orf64_{FL}$) protein ran at a much higher mobility of approximately 300kDa. The predicted size for the full-length, 2636 amino acid protein is 279 kD, which closely correlates with the observed size.

To determine the cellular localization of KSHV Orf64, we transfected the pcDEF3-$Orf64_N$ and pcDEF3-$Orf64_{FL}$ expression plasmids into HEK-293 cells. Forty-eight hours post-transfection, cells were lysed and either separated into cytosolic and nuclear fractions, or harvested as whole cell lysates. As shown in Figure 2B and Figure 2C, the Flag-tagged $Orf64_N$ and $Orf64_{FL}$ proteins were detected in both nuclear and cytosolic fractions. The integrity of the cytosol-nuclear separation was confirmed by blotting for the ER cytoplasmic protein, GRP-78. The signal for GRP-78 was only detectable in the cytoplasmic fraction and the whole cell lysate validating the integrity of the fractionation. These results suggest that both the N-terminus and full-length Orf64 proteins may be found in the cytoplasm and nucleus of the cell.

In vitro deubiquitination Assays. As shown in Figure 1, KSHV Orf64 appears to contain a conserved deubiquitinase domain. In order to determine whether the predicted catalytic core of the Orf64 DUB was functional, we sub-cloned the $Orf64_N$ fragment into pGEX6p-1 to fuse it to GST and express it *in vitro*. GST-$Orf64_N$ was expressed in BL21 bacteria and purified using a GST column to high purity (Fig. 3A). The GST-$Orf64_N$ protein was subsequently used in two different *in vitro* deubiquitinase assays.


A
pcDEF
Orf64N
Orf64FL
300 kD
30 kD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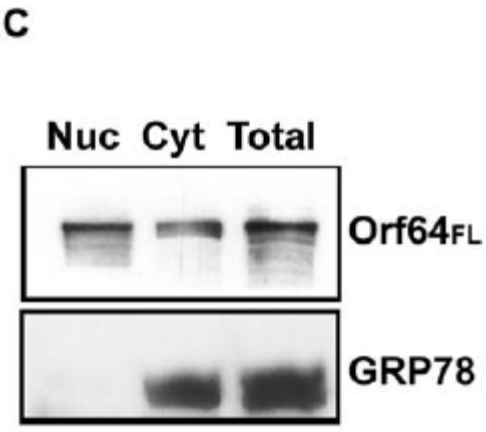
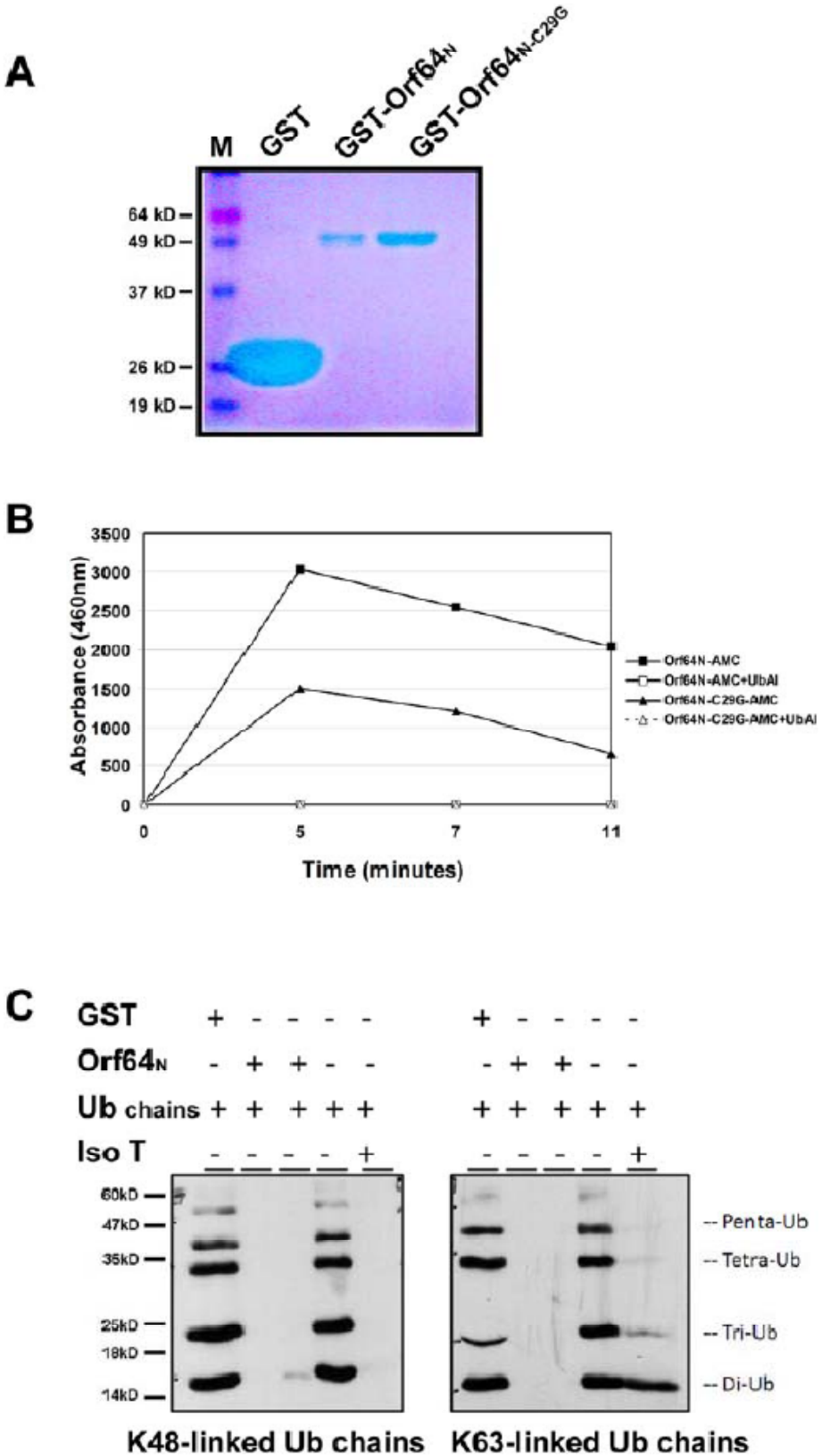

C
Nuc Cyt Total
Orf64FL
GRP78

Figure 2. Expression and intracellular localization of Orf64. A) HEK-293 cells were transiently transfected with expression vectors for Flag-tagged N-terminal Orf64 ($Orf64_N$) and full length ($Orf64_{FL}$). Cells were harvested and the lysates subjected to Western blot analysis with an anti-Flag antibody. $Orf64_N$ displayed a mobility of ~30kD and $Orf64_{FL}$ displayed a mobility of ~300kD. **B)** Transiently transfected cells expressing $Orf64_N$ were subjected to cellular fractionation. The N-terminus of Orf64 was present in both the cytoplasm and the nucleus. Expression of the cytoplasmic marker GRP-78 (lower panel) served as a control for the fractionation. **C)** Transiently transfected cells expressing $Orf64_{FL}$ were subjected to cellular fractionation. The full-length Orf64 protein is also detected in both cytoplasmic and nuclear compartments. Expression of the cytoplasmic marker GRP-78 (lower panel) served as a control for the fractionation.

The first assay used a wild-type ubiquitin substrate. This assay involves the cleavage of the Ubiquitin-AMC substrate by a deubiquitinase to free a fluorophore capable of excitation and emission at 460nm. For this assay, wild-type GST-Orf64$_N$ or a mutant protein in which the cysteine at position 29 was mutated to a Glycine (GST-Orf64$_{N\text{-}C29G}$) was incubated alone, in the presence of the Ubiquitin-AMC substrate, or with substrate and a DUB suicide inhibitor, ubiquitin aldehyde (Ub-Al). The samples were then assayed for fluorescence at a wavelength of 460nm. A graph of the fluorescence values obtained at different time points after treatment is shown in Figure 3B. There is a significant increase in fluorescence in the sample containing GST-Orf64$_N$ protein and Ubiquitin-AMC substrate as compared to GST-Orf64$_N$ without substrate, GST-Orf64$_{N\text{-}C29G}$ with Ubiquitin-AMC, or GST-Orf64$_N$ /GST-Orf64$_{N\text{-}C29G}$ with Ubiquitin-AMC substrate and the Ub-Al suicide inhibitor (Fig. 3B). These data strongly demonstrate that the purified N-terminus of Orf64 is able to cleave synthetic Ub chains *in vitro* in the absence of other cellular proteins, and that the cysteine residue is critical for this activity.

To confirm these findings, a second type of *in vitro* DUB assay was performed. We incubated synthetic, branched K48- or K63-Ub chains with either GST alone or the GST-Orf64$_N$ fusion protein (Fig. 3C). The bonafide deubiquitinase, Isopeptidase T (IsoT) [38], was also used as a positive control in this assay. The samples were run on SDS-PAGE and a Western blot with an anti-ubiquitin antibody was performed. Lanes with Ub chains alone, or Ub chains plus GST protein displayed a laddered pattern corresponding to ubiquitin chains containing different numbers of ubiquitin linkages (Figure 3C). The previously characterized deubiquitinase, IsoT, cleaved all the branched chains (either


A
M
GST
GST-Orf64N
GST-Orf64N-C29G
64 kD
49 kD
37 kD
26 kD
19 kD
B
Absorbance (460nm)
3500
3000
2500
2000
1500
1000
500
0
0
5
7
11
Time (minutes)
Orf64N-AMC
Orf64N-AMC+UbAl
Orf64N-C29G-AMC
Orf64N-C29G-AMC+UbAl
C
GST
Orf64N
Ub chains
Iso T
60kD
47kD
35kD
25kD
18kD
14kD
-- Penta-Ub
-- Tetra-Ub
-- Tri-Ub
-- Di-Ub
K48-linked Ub chains
K63-linked Ub chains

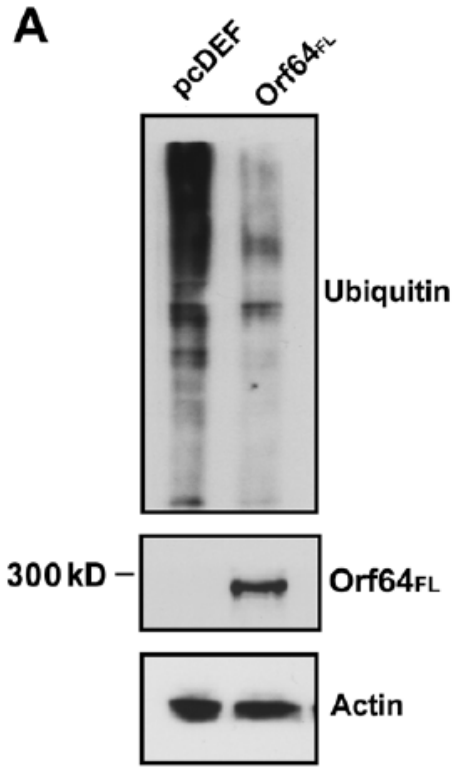
Figure

Figure 3. The N-terminus of Orf64 encodes deubiquitinating (DUB) activity for both K48- and K63-linked ubiquitin chains in vitro. A) GST-Orf64$_{N}$ and GST-Orf64$_{N\text{-}C29G}$ (lanes 2 and 3) were expressed in BL21 (DE) cells with the control GST protein (lane 1). After purification, the proteins were electrophoresed by SDS-PAGE and the gel was stained with Coomasie blue dye. **B)** Purified GST-Orf64$_{N}$ protein was incubated with a wild-type ubiquitin fluorogenic substrate, AMC-Ub. Fluorescence generated from the cleaved AMC-Ub substrate was recorded over time at 460nm. Data from reactions including AMC-Ub with GST-Orf64$_{N\text{-}C29G}$ purified protein, GST-Orf64$_{N}$ plus ubiquitin aldehyde (Ub-Al), or Orf64 $_{N\text{-}C29G}$ plus Ub-Al are also shown. **C)** Purified GST-Orf64$_{N}$ protein was used in another *in vitro* DUB assay with ubiquitin chains. Lysine 48 (K48)- and lysine 63 (K63)-linked chains are not cleaved by GST (lanes 1) or in the absence of GST-Orf64$_{N}$ (lane 4). Ub chains containing K48- and K63-linked ubiquitin moieties were completely cleaved by the positive control, IsopeptidaseT (IsoT, lane 5), as well as in the presence of Orf64$_{N}$ (lanes 2 and 3).

K48- or K63-linkages). Likewise, in the presence of the GST-Orf64$_N$ fusion protein, the K48- and K63-Ub chains were also completely cleaved (Fig. 3C). These results indicate that the N-terminus of Orf64 encodes DUB activity and can cleave both types of Ub linkages, at least as effectively as the IsoT positive control.

In vivo deubiquitination Assays. We next determined whether full-length Orf64 (Orf64$_{FL}$) functioned as a deubiquitinase in a cell-based assay. 2x10^6 HEK-293 cells were plated on 100mm dishes and co-transfected with a plasmid encoding a HA-tagged ubiquitin named pcDNA3.1-3XHA-Ub (WT-Ub) [39], pcDEF3 empty vector, or pcDEF3-Orf64$_{FL}$ plasmids. Cells were harvested 48 hours post-transfection and subjected to Western blot analysis with an anti-HA antibody to detect all ubiquitinated cellular proteins. When pcDEF3-Orf64$_{FL}$ was co-transfected with the WT-Ub expression plasmid, there was very little accumulation of ubiquitinated cellular proteins compared to the control, suggesting that KSHV Orf64 $_{FL}$ can cleave ubiquitin moieties off cellular proteins (Fig. 4A). We also performed identical assays with Orf64$_N$ in HeLa, CV-1 cells and the KSHV-positive rKSHV.219-Vero cells, and found that Orf64$_N$ retained the deubiquitinating activity of Orf64 in all these cells lines, i.e. in the presence or absence of the viral genome (Fig. 4B).

In order to identify which ubiquitin linkage type is targeted by Orf64 *in vivo*, we co-transfected Orf64$_{FL}$ or empty vector with HA-tagged WT-Ub, or a plasmid (K48-Ub) in which all lysines in ubiquitin were mutated except for lysine 48, or another plasmid (K63-Ub)in which only lysine 63 was left intact. The latter two constructs produce ubiquitin chains that can only be polymerized through their K48 or K63 lysine residues, respectively. Cells were harvested forty-eight hours post-transfection and subjected to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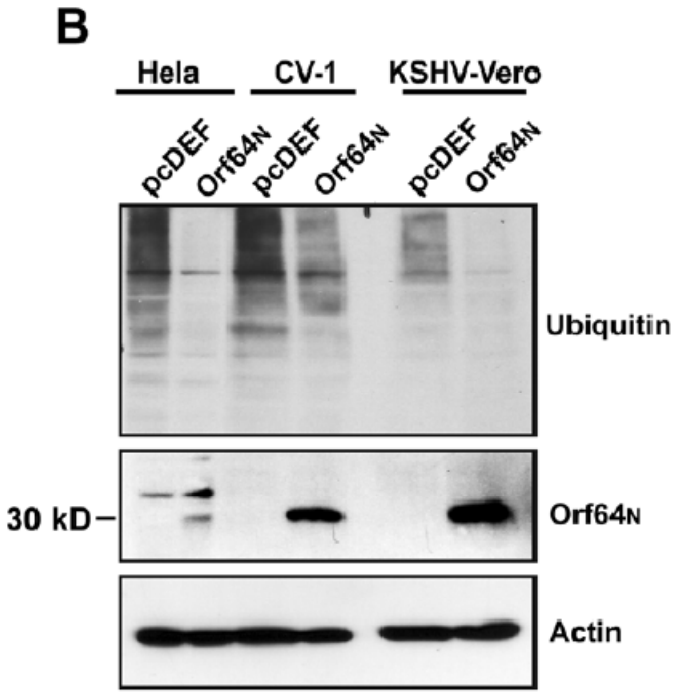
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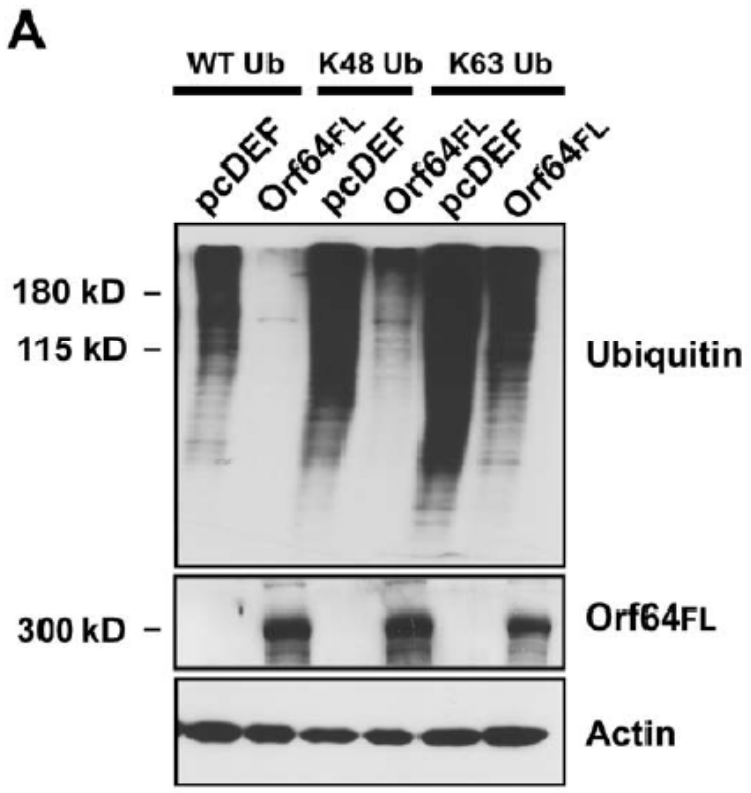
Figure 4. ***Orf64 exerts DUB activity in vivo.*** **A)** Co-transfection of pcDEF3-Orf64$_{FL}$ and HA-tagged ubiquitin expression plasmids in HEK-293 cells. Cells were harvested 48 hours post-transfection and subjected to Western blot analysis with an anti-HA antibody. Expression of Orf64$_{FL}$ protein results in decreased levels of high molecular weight ubiquitinated cellular proteins as compared to the empty vector (pcDEF) control. Western blots for actin show equal loading of protein lysates and western blots with anti-Flag antibody show expression of the full-length Orf64 protein. **B)** Co-transfection of pcDEF3-Orf64$_{N}$ and HA-tagged ubiquitin expression plasmids in HeLa, CV-1 and KSHV-positive rKSHV.219-Vero cells (KSHV-Vero). Cells were harvested 48 hours post-transfection and subjected to Western blot analysis with anti-HA antibody. Expression of Orf64$_{N}$ protein results in decreased levels of high molecular weight ubiquitinated proteins as compared to the empty vector (pcDEF) control. Thus, the DUB activity is encoded within the N-terminal 205 residues of Orf64. Western blots for actin show equal loading of protein lysates and western blots with an anti-Flag antibody show expression of Orf64$_{N}$ protein.

Western blot analysis with an anti-HA antibody to detect the presence of ubiquitinated proteins in the cell. We found that lysates from cells transfected with the different ubiquitin expression constructs and empty vector (pcDEF) showed accumulation of many ubiquitinated cellular proteins. However, full-length Orf64 ($Orf64_{FL}$) exerted a significant deubiquitination effect on cellular proteins (Fig. 5A) and displayed a deubiquitinase preference (but not specificity) towards K48-mediated ubiquitin linkages. KSHV $Orf64_{FL}$ was able to deubiquitinate most of the K48-linked ubiquitinated proteins and a significant proportion of the K63-linked ubiquitinated proteins (Fig. 5A).

Next, we determined whether $Orf64_N$ displayed the same linkage-type specificity *in vivo*. Notably, both K48- and K63-linked ubiquitin chains were completely cleaved by $Orf64_N$ with no apparent preference in the HEK-293 transfected cells (Fig. 5B). We also transfected HEK-293 cells with WT-Ub and either the wild-type N-terminal Orf64 ($Orf64_N$) or mutant N-terminal ubiquitin antibody to detect ubiquitinated proteins. We found that wild-type $Orf64_N$ displayed deubiquitinase activity against cellular ubiquitinated proteins but that the mutant $Orf64_{N\text{-}C29G}$ protein, showed a loss of DUB activity compared to the wild type $Orf64_N$ protein (Fig. 5C). In addition, a full-length clone containing the C29G mutation ($Orf64_{FL\text{-}C29G}$) also displayed inhibition of DUB activity compared to the wildtype full-length clone ($Orf64_{FL}$) when introduced into cells expressing WT-Ub (Fig. 5D). These results indicate that the predicted N-terminal catalytic core of KSHV Orf64 is functional and the cysteine 29 residue is indeed required for the DUB activity of this protein.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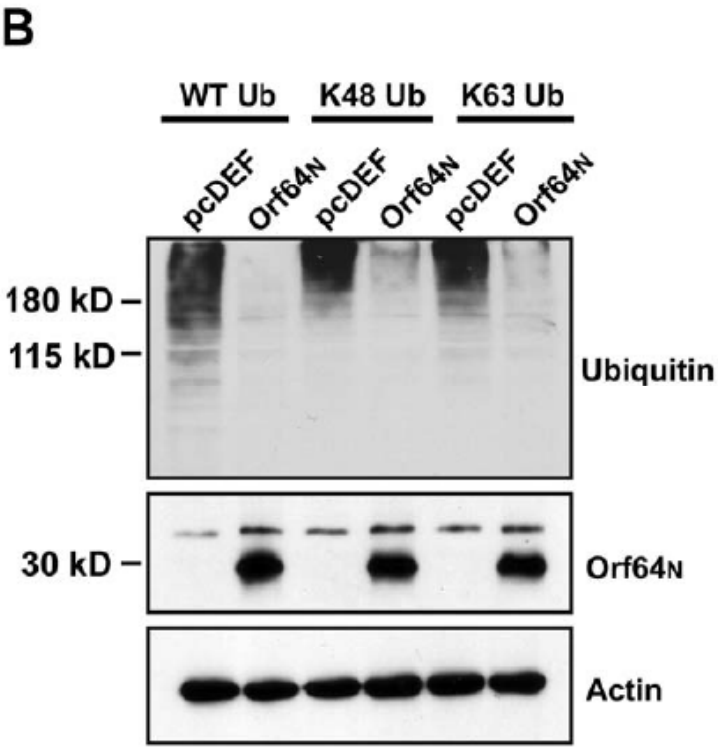
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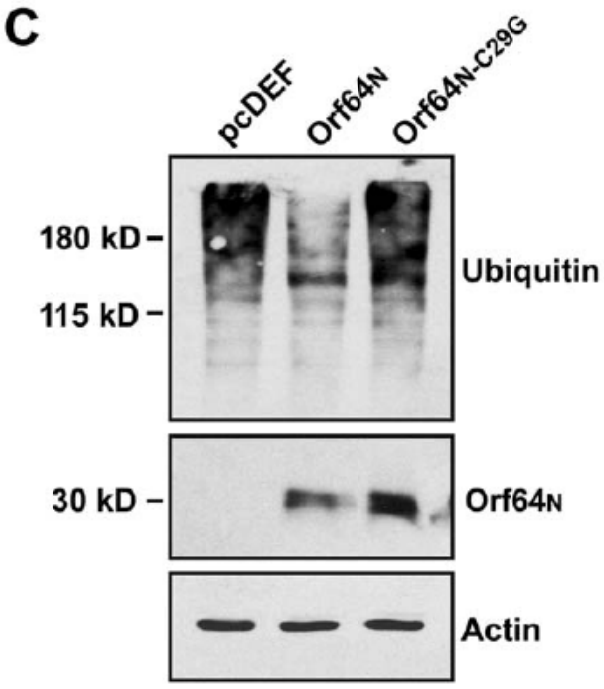
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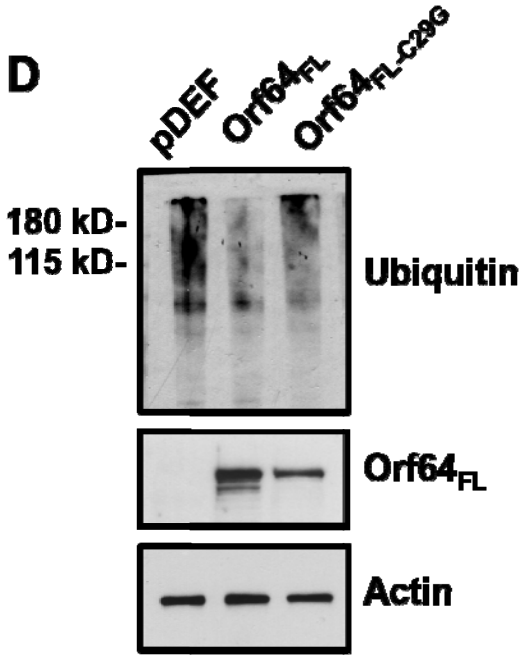
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Figure 5. ***Orf64 can effectively cleave both K48 and K63 ubiquitin linkages.*** **A)** pcDEF3 or pcDEF3-Orf64$_{FL}$ expression plasmids were co-transfected with expression plasmids for WT-Ub, K48-Ub, or K63-Ub. Cells were harvested 48 hours post-transfection and subjected to Western blot analysis with an anti-HA antibody. Orf64$_{FL}$ was able to deubiquitinate WT-, K48- and K63-linked ubiquitinated cellular proteins. Actin blots show equal loading of protein lysates and a Western blot with an anti-Flag antibody shows expression of Orf64$_{FL}$ protein. **B)** pcDEF3 or pcDEF3-Orf64$_{N}$ expression plasmids were co-transfected with expression plasmids for WT-Ub, K48-Ub, or K63-Ub. Cells were harvested 48 hours post-transfection and subjected to Western blot analysis with an anti-HA antibody. The N-terminal Orf64$_{N}$ protein was able to deubiquitinate WT-, K48- and K63-linked ubiquitinated cellular proteins. Western blots for actin show equal loading of protein lysates and a Western blot with an anti-Flag antibody shows expression of the Orf64$_{N}$ protein. show equal loading of protein lysates and a western blot with an anti-Flag antibody shows expression of both the Orf64$_{N}$ and Orf64$_{N\text{-}C29G}$ proteins. **C)** Co-transfection of pcDEF3, pcDEF3-Orf64$_{N}$ and pcDEF3-Orf64$_{N\text{-}C29G}$ with WT-Ub expression plasmids in HEK-293 cells. Cells were harvested 48 hours post-transfection and subjected to Western blot analysis with an anti-HA antibody. Orf64$_{N}$ can deubiquitinate cellular proteins but the deubiquitinase activity of Orf64$_{N}$ is ablated by the C29G mutation suggesting that this cysteine is essential for deubiquitination mediated by Orf64.

(Continued…)

D) Co-transfection of pcDEF3, pcDEF3-Orf64$_{FL}$ and pcDEF3-Orf64$_{FL-C29G}$ with WT-Ub expression plasmids in HEK-293 cells. 48 hours post-transfection lysates were blotted with an anti-HA antibody. Orf64$_{FL}$ can deubiquitinate cellular proteins but the deubiquitinase activity is dependent on the C29 residue as demonstrated by the C29G mutation suggesting that this cysteine is essential for deubiquitination mediated by Orf64. Western blots for actin show equal loading in all panels.

Thus, in summary, our data suggests that KSHV Orf64 is a potent deubiquitinase against ubiquitinated cellular proteins and that both K48- and K63-linked ubiquitinated proteins can be deubiquitinated by KSHV Orf64. Additionally, our data suggests that the first 205 amino acids of KSHV Orf64 are sufficient for this activity.

Effect of Orf64$_{FL}$ on KSHV lytic promoters. In an effort to functionally characterize KSHV Orf64 to a greater extent, and because this protein can localize to the nucleus (Figure 2), we performed luciferase assays to identify any effects Orf64 may exert on lytic promoters of KSHV. HEK-293 cells were transfected with empty vector, pcDNA3-KSHV Orf50 (RTA), pcDEF3-Orf64$_{FL}$ or both pcDNA3-KSHV Orf50 (RTA) and pcDEF3-Orf64$_{FL}$ plasmids in addition to individual lytic promoter-driven luciferase plasmids. The lytic promoters examined were vGPCR, glycoprotein B (gB) DNA-binding protein (DBP), RTA, LANA, and Orf57 as previously described [40]. The luciferase values were normalized to β-galactosidase and expressed as fold induction over empty vector. As shown in Figure 6, full-length KSHV Orf64 does not directly affect transcription from viral lytic promoters. However, in the presence of KSHV RTA, Orf64 can enhance RTA-mediated induction of a subset of lytic promoters. For the gB, RTA, Orf57 and vGPCR promoters, Orf64 increased RTA transactivation approximately 2-3 fold over RTA levels, which were already in the range of 10- and 75-fold over empty vector (Fig. 6).

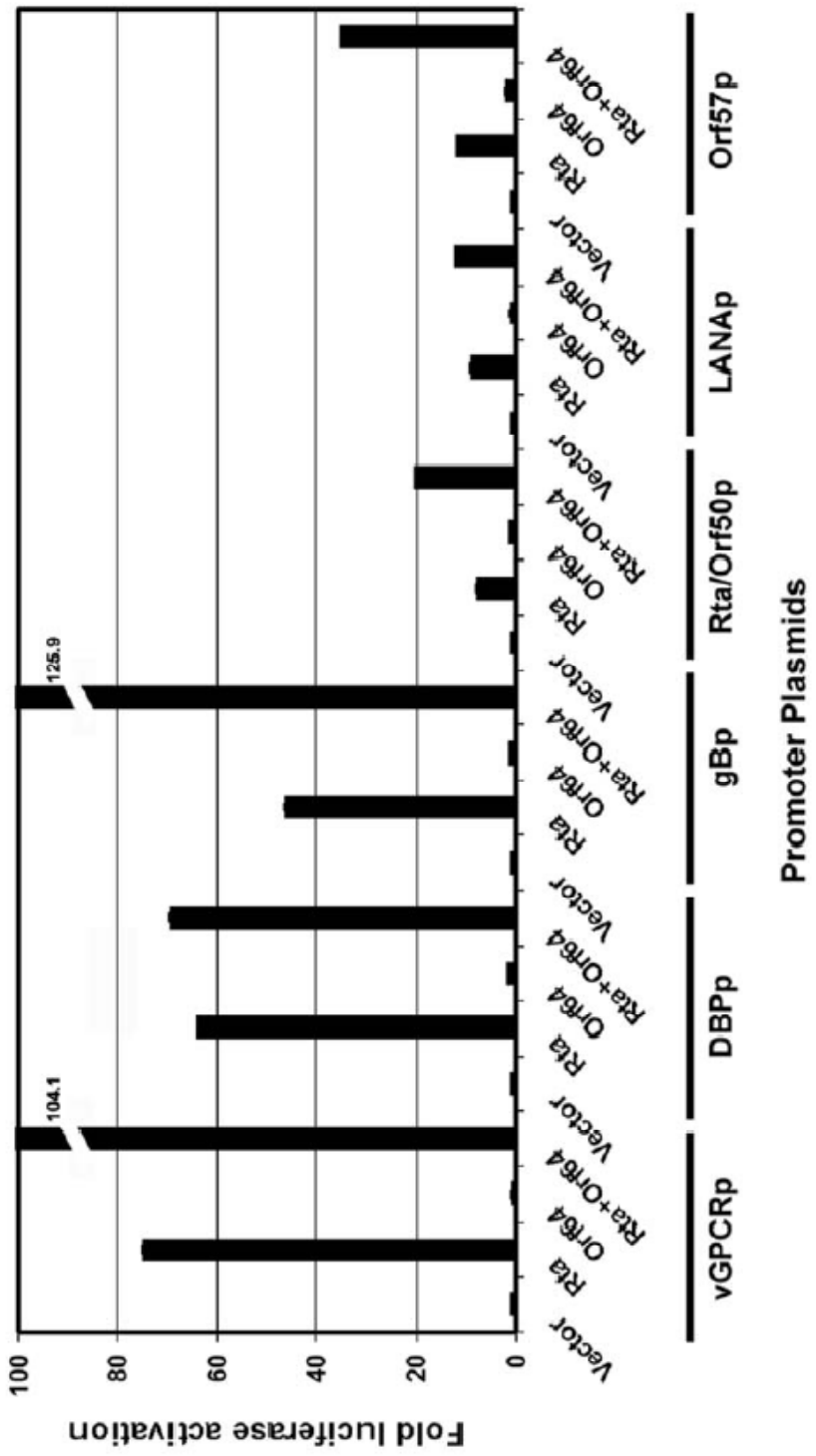
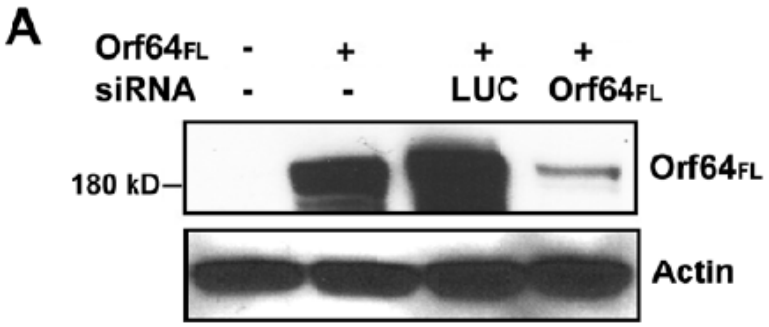

Fold luciferase activation
100
80
60
40
20
0
104.1
125.9
Vector
Rta
Orf64
Rta+Orf64
vGPCRp
DBPp
gBp
Rta/Orf50p
LANAp
Orf57p
Promoter Plasmids

Figure 6. ***KSHV Orf64 can enhance RTA-mediated transcriptional activation of lytic promoters.*** Empty vector (pcDEF3), RTA, $Orf64_{FL}$, or RTA plus $Orf64_{FL}$ expression constructs were cotransfected into HEK-293 cells with a ß-galactosidase expression plasmid and promoter-luciferase reporter plasmids in which the KSHV vGPCR, DBP, gB, RTA, LANA, and Orf57 lytic promoters drive expression of firefly luciferase. Forty eight hours post-transfection, promoter activity was determined by luciferase assay. Luciferase activities were normalized to ß-galactosidase and expressed as fold induction over empty plasmid. Each experiment was performed in triplicate and repeated at least four times.

Orf64 and lytic replication. Since Orf64 showed the capacity to enhance RTA-induced lytic promoter activation, we hypothesized that it may contribute to the early stages of the lytic replication cycle. In order to identify the contribution of Orf64 to the lytic cascade, we designed short interfering RNAs (siRNAs) targeting Orf64 for degradation via the RNA-induced silencing complex (RISC). To test the specificity and effectiveness of the Orf64 siRNA knockdown, we transfected HEK-293 cells with the pcDEF3-Orf64$_{FL}$ expression plasmid along with either an irrelevant siRNA directed against luciferase (LUC) or the Orf64-specific siRNA (Orf64$_{FL}$ siRNA) (Dharmacon). Figure 7A shows the extent and specificity of the Orf64-directed siRNA at knocking down Orf64$_{FL}$ protein levels as measured by a Western blot to detect the presence of Orf64. Using this Orf64 siRNA we were able to consistently achieve a robust knockdown of the exogenously expressed Orf64$_{FL}$ protein. Actin was used as a control and showed no difference in protein expression in all four lanes (Fig. 7A).

KSHV-infected latent 293 cells (KSHV-293) were made by infecting HEK-293 cells with the rKSHV.219 virus [41] and selecting with 1μg/ml puromycin. We subsequently induced the KSHV-293 cells into lytic reactivation by infecting with baculovirus encoding KSHV-Orf50 (Bac50) and 1.7mM sodium butyrate. A control mock induced sample was also included using media without Bac50 or sodium butyrate. Twenty-four hours post-induction, we transfected cells with either LUC- or Orf64-directed siRNAs. Cells were harvested for total RNA analysis as well as protein analysis at 48 hours post-transfection. The RNA was used in reverse-transcription (RT) reactions. The resulting cDNAs were then used as templates for PCR amplification with Orf64, Orf57 (early lytic gene) and GAPDH primers to measure viral transcript levels and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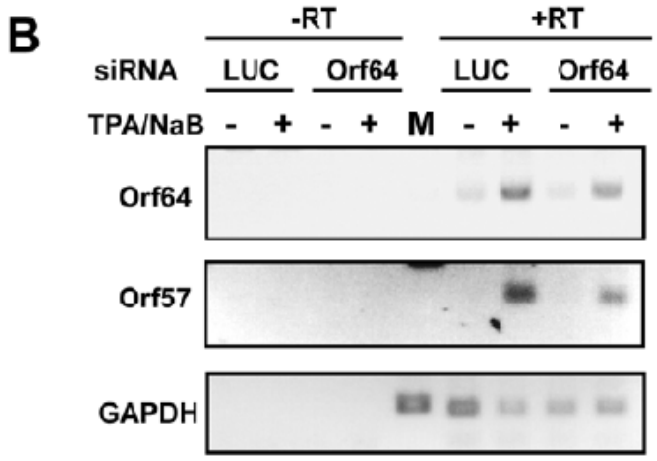
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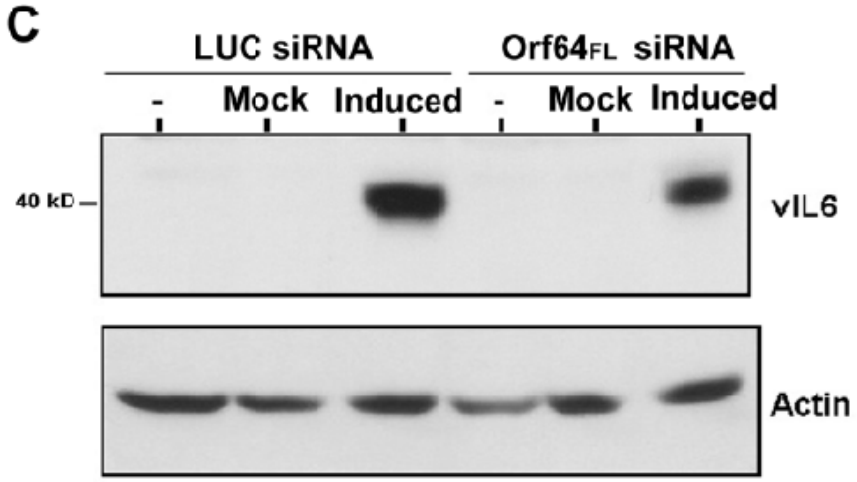
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Figure 7. ***Knock-down of Orf64 affects transcription and protein expression of lytic genes.*** **A)** HEK-293 cells were transfected with pcDEF3-Orf64$_{FL}$ and either siRNA against Orf64 or siRNA against luciferase (LUC). Cells were harvested 48 hours post-transfection and subjected to Western blot analysis with an anti-Flag antibody to detect Orf64$_{FL}$ expression. Knockdown of exogenous Orf64$_{FL}$ in HEK-293 cells was observed when a siRNA specific to Orf64, but not luciferase (LUC siRNA), was co-transfected with the Orf64$_{FL}$ expression vector. An actin blot was performed to show equal loading of protein lysates. **B)** KSHV-293 cells were induced (or mock-induced) into lytic replication with Bac50 and sodium butyrate, and 24h later transfected with siRNAs against Orf64 or luciferase (LUC). Total RNA was harvested 48h post-transfection and used in reverse-transcription PCR reactions. The upper panel shows knockdown of the Orf64 transcript while the middle panel shows a reduction in the levels of Orf57 transcript. A GAPDH amplification was also performed as a control (bottom panel). The reactions performed without reverse-transcriptase (-RT lanes), result in no detectable product demonstrating the absence of DNA contaminants in the RT-PCR reactions. **C)** Protein lysates from untreated, mock-induced, and induced cells were analyzed by Western blot for the levels of the KSHV lytic protein, vIL6. An actin blot was also performed to show equal loading of protein lysates.

determine levels of reactivation. As shown in Figure 7B, moderate knockdown of the endogenous Orf64 transcript resulted in decreased Orf57 transcription in the reactivated KSHV- 293 cells, while GAPDH transcript levels remained unchanged. This suggests that knockdown of Orf64 results in decreased lytic reactivation of KSHV.

To confirm these findings, we also performed Western blot analysis with an anti-viral IL-6 (vIL6) antibody (Fig. 7C). We found that knockdown of Orf64 also resulted in decreased levels of vIL6, an early lytic protein, while cellular actin levels remained unchanged (Fig.7C).

DISCUSSION

Sequence alignments indicate that all known herpesviruses encode a large tegument protein which is often the largest open-reading frame in the whole genome. Studies on HSV-1, PRV and CMV have suggested several distinct roles for this herpesviral core protein. These roles include, but are not limited to, delivery of genomic DNA into the nucleus, egress from the nucleus, and scaffolding during tegument assembly [29,30,33]. One unifying role of the Orf64 homologs common to all herpesviruses studied thus far is the ubiquitin specific protease (USP) or deubiquitinase (DUB) activity encoded in the N-terminus of these proteins. This activity has been found to affect plaque size, titers, and neuroinvasiveness of PRV, but has been deemed non-essential *in vivo* for PRV, yet required *in vitro* [30,31]. In contrast, in the case of HSV-1, UL36 is indispensible for transport along microtubules. Despite these differences it is clear that UL36 homologs in the alpha herpesviruses play pivotal, if not required, roles in the lytic lifecycle. Furthermore, the targets for the herpesviral DUBs have not yet been identified.

We are the first to report the functional characterization of KSHV Orf64-encoded deubiquitinase. We found that the full-length Orf64 protein showed both nuclear and cytoplasmic localization indicating that Orf64 protein shuttles between both these cellular compartments. In two different sets of *in vitro* deubiquitinase assays, we found that a GST-$Orf64_N$ fusion protein was efficiently able to cleave ubiquitin linkages and did not display preference for K63- or K48-linked ubiquitin chains. Furthermore, expression of both the N-terminus and full-length Orf64 proteins in different cell types resulted in a marked reduction of high molecular weight ubiquitinated proteins, suggesting that Orf64 can deubiquitinate cellular proteins *in vivo*. Moreover, an N-terminal mutant Orf64 protein in which the putative cysteine catalytic residue was mutated to glycine ($Orf64_{N\text{-}C29G}$) was devoid of DUB activity. In a similar fashion, a full-length Orf64 clone encoding the C29G mutation ($Orf64_{FL\text{-}C29G}$) was also deficient in DUB activity compared to wild type full-length Orf64 ($Orf64_{FL}$). The most likely explanation for the requirement of this single amino acid is that C29 is part of the catalytic core or catalytic triad that defines the cysteine protease active site as predicted by sequence analysis (Fig 1). In sum, our data suggest the following: First, KSHV Orf64 encodes a robust deubiquitinase enzyme. Second, the DUB activity is encoded within the first N-terminal 205 residues. Third, the cysteine residue at position 29 is critical for DUB activity. Fourth, the KSHV Orf64 protein does not display marked specificity towards K48- or K63-linked ubiquitin chains, although the deubiquitination of K48-linked ubiquitin chains appears to be slightly favored. The ability of KSHV Orf64 to deubiquitinate both K48 and K63 linkages is unique, since all other herpesviral DUBs target K48 linkages specifically [36,42].

The ability of KSHV Orf64 to enter the nucleus and its sequence homology with other herpesviral DUBs suggests a role in virion assembly and DNA packaging. Additionally, its presence in the nucleus suggested that it might affect viral transcription, although other herpesviral DUB homologs have not been tested for effects on transcriptional regulation. We performed promoter reporter assays and found no direct effect of Orf64 on lytic promoters. However, the Orf64-encoded protein was able to synergize with RTA/Orf50 to increase the level of transcription from RTA-responsive promoters. The promoters for vGPCR, gB, RTA, and Orf57 showed increased activity in the presence of RTA and Orf64, compared to RTA alone. Glycoprotein B (gB) is a late protein which is present on the viral envelope and involved in viral entry [43]. Upregulation of this protein could enhance both the infectivity and amount of virions released. The vGPCR protein plays a role in promoting cellular proliferation and angiogenesis by inducing the release of vascular endothelial growth factor (VEGF) into the microenvironment [44]. Upregulation of transcripts for the RTA and Orf57 proteins, both of which can induce transcription from the RTA promoter [45,46], suggest a role for Orf64 in supporting lytic replication by positively regulating RTA, the lytic switch protein. Our data suggests that Orf64 could be synergizing with RTA to maintain the lytic state of replication. To our knowledge, this constitutes the first report of a herpesviral deubiquitinase exerting any effect on viral promoters. Furthermore, we found that siRNA knockdown of Orf64 in KSHV-infected 293s induced to enter the lytic cycle, resulted in decreased levels of Orf57 lytic transcripts as well as decreased expression of the lytic protein, vIL6. This suggests that Orf64 expression can enhance the KSHV lytic cycle, most likely through its deubiquitination function.

The identification of KSHV Orf64 as a robust deubiquitinase that can catalyze cleavage of both K48- and K63-linked ubiquitin sets it apart from all its known herpesviral homologs. Furthermore, the synergistic increase in lytic promoter activation indicates that Orf64 may play a novel role in enhancing lytic replication, distinct from its predicted roles in tegument and virion assembly. Moreover, Orf64 is present in the tegument of the incoming virion during *de novo* infection [34]. Thus, it is in a position to exert an effect on KSHV replication upon entry of the virus into the cell. This provides a window of opportunity whence Orf64 could help to modify the cellular environment in order to precipitate the onset of lytic replication, maximize its efficiency, or both.

ACKNOWLEDGMENTS

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CHAPTER FOUR

MULTIPLE FUNCTIONS OF ORF64

Carlos M. González and Blossom Damania

ABSTRACT

Ubiquitin-mediated regulation of cellular signaling pathways is emerging as a regulatory mechanism that rivals phosphorylation in its breadth and complexity. One signaling pathway known to be regulated by ubiquitination is the NF-κB pathway. Several reports implicate NF-κB signaling in both latency and lytic replication of KSHV-infected cells. We have recently described a deubiquitinase (DUB) encoded by KSHV Orf64. Given the potent deubiquitination potential of KSHV Orf64 and the requirement of ubiquitin regulation for NF-κB signaling, we asked whether Orf64 could deregulate NF-κB pathway signaling. KSHV Orf64 expression induced the degradation of IκB, an inhibitor of NF-κB signaling whose degradation triggers the activation of the pathway. In addition, lytic reactivation of KSHV-infected cells was also shown to reduce the levels of IκB, indicating that NF-κB signaling is activated upon lytic replication in endothelial cells. These results suggest that Orf64 may play a novel role in viral replication via the activation of NF-κB signaling. Because Orf64 is present in the incoming virion it is in a position where it could induce NF-κB activation before the onset of viral transcription, setting the stage for the successful establishment of viral infection.

INTRODUCTION

As mentioned in the previous chapter, the Ubiquitin (Ub) system represents a protein regulatory mechanism of the cell. Ubiquitin is widely expressed and conserved. The ubiquitin molecule is post-translationally conjugated to specific target proteins to regulate their stability and function. Cells use Ub to manipulate a variety of cellular processes including protein expression, apoptosis, cell cycle regulation, receptor trafficking, DNA repair, and signal transduction and antigen presentation [1,2,3].

There is ample evidence that viruses have targeted the Ub system for subversion in order to forward the viral agendas of replication and survival. Herpesviruses in particular have usurped this mechanism by encoding Ub-regulatory molecules in their genomes. These include proteins that act as Ub conjugating molecules or E3 ligases as well as Ub-deconjugating proteins or deubiquitinases (DUBs). The DUBs, in particular, are part of a set of genes that seem to have been encoded in herpesviral genomes for a long evolutionary time and are termed 'core' genes [4]. These genes have been shown to encode large, multifunctional tegument proteins decorating the viral capsid [5].

As discussed in chapter 3, we have recently functionally described KSHV Orf64 as a robust deubiquitinase (DUB) as measured by *in vitro* as well as *in vivo* deubiquitination assays ([6], submitted). KSHV Orf64 encodes the DUB activity in the N-terminus and unlike other herpesviral DUBs, which show specificity for K48 ubiquitin linkages [7,8], KSHV Orf64 appears to deubiquitinate both K48- and K63-linked ubiquitin chains. Its ability to localize to both the nuclear and cytoplasmic compartments may be tied to another novel function of this DUB i.e. the capability to modulate transactivation of RTA-responsive promoters in reporter assays.

We also found that siRNA knockdown of KSHV Orf64 resulted in decreased reactivation from latency and decreased viral replication suggestive of an important role of Orf64 in replication. Moreover, the ability of KSHV Orf64 to deubiquitinate a wide array of cellular proteins provides the virus with a large number of potential DUB targets, including transcriptional activators, with previously unidentified effects on both viral replication and cellular signaling.

Cellular responses to environmental stimuli are mediated by inducible transcription factors and are mediated to a significant extent through changes in gene expression. A key player in responding to environmental changes is nuclear factor (NF-κB), an evolutionarily conserved signaling molecule that plays a critical role in a variety of cellular processes. NF-κB plays an important role in the immune system [9,10,11] but also regulates gene expression for other biological processes and can thus influence multiple aspects of both normal and disease states.

There are five members in the NF-κB family, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), p65 (RelA), RelB, c-Rel. In resting cells they exist as cytoplasmic homo- or heterodimers. Proteins of the IκB (inhibitor of κB) family bind to NF-κB to form a complex that prevents NFκB translocation to the nucleus where it exerts its activity. NF-κB signaling is characterized as proceeding through either the classical or alternative pathway [9]. The classical or canonical pathway is activated by stimulation with pro-inflammatory cytokines, pathogenic stimuli or antigenic peptides. Stimulation of the classical pathway results in the activation of the β subunit of the IκB kinase (IKK) complex, which can phosphorylate IκB

proteins. The NF-kB p105 subunit is processed into p50 which can heterodimerize with RelA (p65) or form p50/p50 homodimers.

In the alternative or non-canonical pathway, the α subunit (IKKα) is activated instead. IKKα can then phosphorylate p100. Phosphorylation of IκB proteins induces lysine 48 (K48)-linked polyubiquitination by the Skp1–Cullin1– F-box (SCF)/-transducin repeat-containing protein (TrCP) E3-ligase complex resulting in proteasomal processing by the proteosome. Ubiquitin/proteosome-mediated processing releases p50-, p65- and c-Rel-containing heterodimers. The resulting subunits form NF-κB complexes which then translocate to the nucleus, where they bind to NF-κB-responsive sequences in the promoters of target genes to drive transcription (Fig. 1).

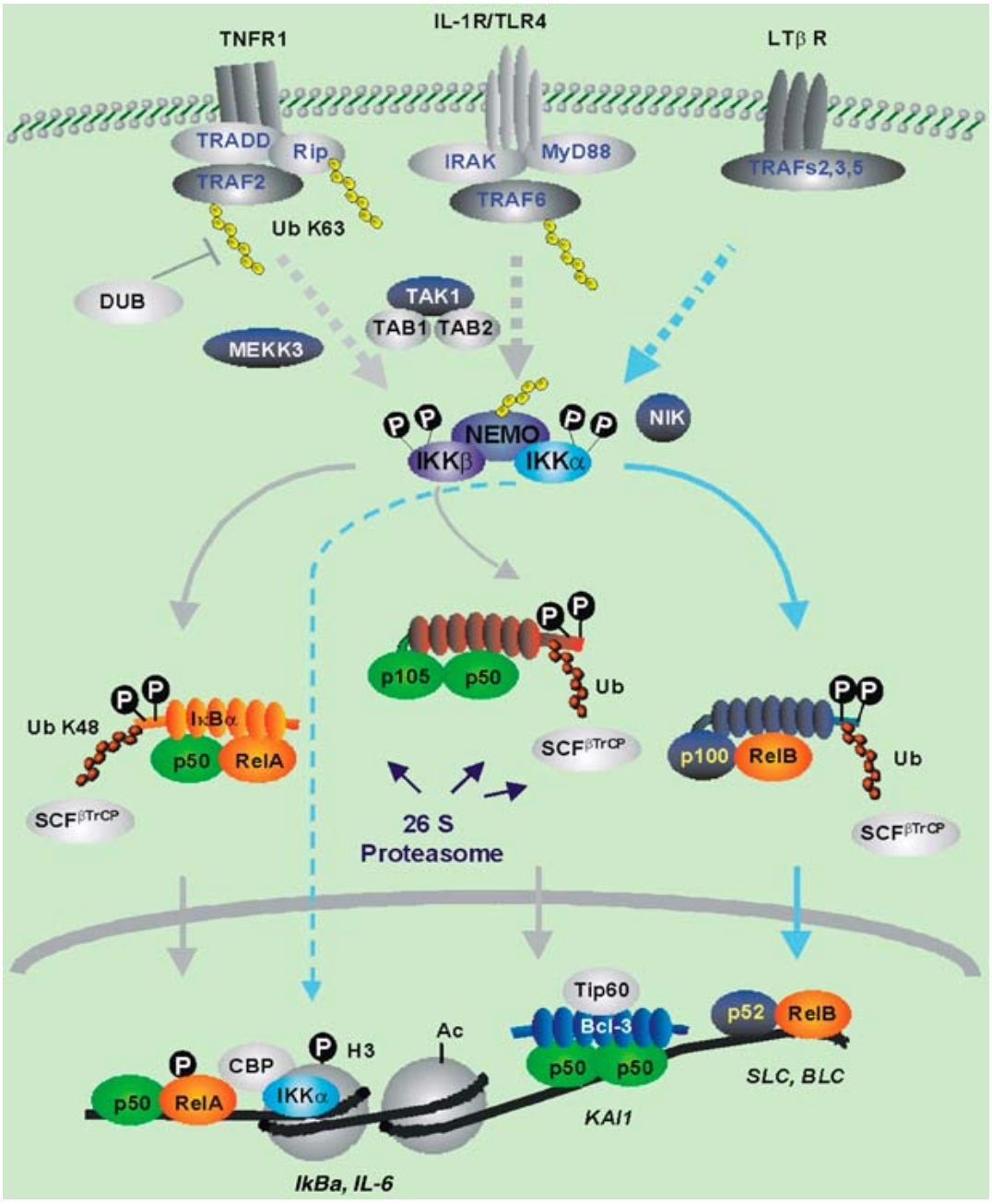
The IκB family is made up of seven members: IκBα, IκBβ, IκBγ, IκBε, BCL-3, and precursor proteins p100 and p105. The degradation of IκB proteins is a tightly regulated process that starts with phosphorylation by the activated IKK complex. The large IKK (900kDa) complex has been shown to contain two kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, termed NEMO (NF-κB essential modifier) or IKKγ [12]. Several recent studies indicate that the Ub system may play a pivotal role during IKKs activation after tumor necrosis factor- (TNF-α), interleukin 1 (IL-1), lipo-polysaccharide (LPS) stimulation as well as antigen presentation. Indeed, IKKγ, considered the 'molecular switch of NF-κB activation', can be polyubiquitinated through lysine residues 399, 285, 277 and 309 [13,14,15].

Purified IKK complex has been shown to require functional TRAF-6 (TNF receptor (TNFR)-associated factor 6), a lysine-63 (K63)-specific E3 ligase [16]. IL-1 receptor (IL-1R)

and Toll-like receptor (TLR) signaling are both dependent on TRAF-6 mediated, K63-linked polyubiquitination of IKKγ [17,18]. TRAF-2 can also catalyze K63-linked polyubiquitination of RIP (receptor-interacting protein) [19]. In TNF-α-stimulated cells RIP-1 is essential during receptor complex formation. Interestingly, a study with TRAF2/TRAF5 doubly deficient mice [20] showed a severe impairment in TNF receptor (TNFR)-induced NF-κB activation. This suggests that TRAF2 and TRAF5 function in TNF signaling in a similar fashion to TRAF6 during IL-1 activation.

TGF-β-activated kinase 1 (TAK1) is recruited to TRAF6 on IL-1 signaling and activates the IKK/NFκ-B pathway [21]. TAK1-binding (TAB) proteins stimulate TAK1 kinase activity by binding to it.TAB2 and TAB3 are the molecular receptors for K63-modified substrates of TRAF2 and TRAF6 [18].

The K63-specific DUB CYLD was identified by screening for DUBs that would down-modulate NF-κB signaling and due to its association with IKKγ [22,23,24]. Mutations in the CYLD gene cause familial cylindromatosis, a rare autosomal-dominant disease. Patients who suffer from cylindromatosis develop benign tumors from the proliferative cell types of hair follicles or eccrine glands. Expression of CYLD is induced by NF-κB, exerting autoregulatory feedback loop that limits IKK activity [25]. CYLD interferes with IKK/NF-κB signaling by selectively cleaving K63-linked Ub chains from TRAF2, TRAF6 and IKKγ, but not K48-linked Ub chains on IκB. CYLD can repress TNF and IL-1 -mediated NF-κB activation when overexpressed.


TNFR1
IL-1R/TLR4
LTβ R
TRADD
Rip
TRAF2
IRAK
MyD88
TRAF6
TRAFs2,3,5
Ub K63
DUB
TAK1
TAB1
TAB2
MEKK3
NEMO
IKKβ
IKKα
NIK
p105
p50
Ub
SCFβTrCP
Ub K48
IκBα
p50
RelA
SCFβTrCP
26 S
Proteasome
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RelB
Ub
SCFβTrCP
Tip60
Bcl-3
p50
p50
p52
RelB
SLC, BLC
KAI1
p50
RelA
CBP
IKKα
H3
Ac
IkBa, IL-6

Figure 1. Diagram of the canonical and non-canonical NFκB signaling pathways and main components. Several membrane receptors stimulate the canonical pathway via adaptors (e.g. TRAFs), which results in IKK complex activation. K63-linked, non-degradative ubiquitination of TRAFs, RIP and NEMO/IKKγ, binding of ubiquitin chains by TABs define TABs or NEMO/IKKγ and phosphorylation of IKKα and β are required for IKK activation. Deubiquitinating enzymes (DUB) counter Ub-mediated regulation. The IKK complex phosphorylates IκBs and p105, which are modified with K48-linked Ub chains by $SCF^{\beta TrCP}$ and targeted to the proteosome. The result is heterodimers of p50 with p65, or p50 homodimers. LTβ and BAFF can also trigger p100 processing by way of TRAFs, NIK kinase and IKKα-containing complexes. p100 is phophorylated, ubiquitinated and processed to p52. p52 heterodimerizes with RelB. The yellow circles represent K63-linked ubiquitin and the red circles denote K48-linked ubiquitin. (Taken from Scheidereit, 2006.)

A20 is another recently described DUB that interferes with TNF-α-induced NF-κB responses [26]. A20 has been shown to downregulate IKK activation by two distinct mechanisms. The N-terminal domain of A20 encodes DUB activity that can cleave K63 chains from RIP and TRAF6, blocking TNFR and TLR signaling to NF-κB, respectively [19,27]. The C-terminal contains a zinc-finger domain that serves as a K48-specific Ub E3 ligase that can catalyze the ubiquitination of RIP which directs it towards proteasomal degradation [19,27]. Recently, Cezanne and TRAF-binding domain (TRABID) have been identified as two other potential DUBs that may interact with TRAF6 [28]. Overexpressing Cezanne results in downregulation of NF-κB, indicating that Cezanne may block IKK activation.

There are several conflicting reports on the effects of KSHV infection on the NF-κB pathway activation status. Initially it was reported that pharmacological inhibition of NF-κB with Bay 11-7082 (Bay-11) resulted in increased levels of lytic reactivation in PEL cells [29]. The same study also found that KSHV lytic promoter induction is inhibited by overexpression of p65. Moreover, latently infected cells express v-FLIP which induces NF-κB activation via interactions with IKKγ and [30,31] activation of the IKK complex. Additionally, reports of PEL cells entering apoptosis upon treatment with Bay-11 have also surfaced [32] and suggest that NF-κB activity is necessary for survival of KSHV-infected PEL cells, contrary to the aforementioned reports. However, apoptosis due to late stage viral infection was not ruled out in this report. Furthermore, it has also been shown that human fibroblasts are not induced into lytic reactivation upon inhibition of the NFκB pathway and that NFκB is activated during lytic replication in several cell types tested [33].

These findings coupled with the dependence of the NF-κB pathway on Ub-mediated regulation led us to investigate whether KSHV ORF64 play a role in the regulation of NF-κB pathway activation on its own and during lytic reactivation.

MATERIALS AND METHODS

Cell Culture: HEK-293 cell lines were maintained in complete media: Dubelcco's Modified Eagle Media (DMEM) (Cellgro) with 10% fetal bovine serum (FBS), L-glutamine, and penicillin plus streptomycin. KSHV-293 cells were established by overlaying supernatants from reactivated rKSHV.219-Vero on naive 293 cells followed by puromycin selection at 1μg/ml. All cells were grown in a 37°C incubator, with 5% CO_2.

Induction of KSHV-293 cells (K293) was achieved by infecting with baculovirus encoding the Orf50 gene (RTA) of KSHV for one hour at 37°C. The inoculum was removed and replaced with complete medium in the mock-induced set, or complete medium with 1.7mM Sodium Butyrate in the induced set. Seventy-two hours post-induction cell lysates were harvested as described below and subjected to western blot analysis.

Western blot analysis: Cells in 100mm dishes were transfected using Superfect reagent (Qiagen). Cells were harvested 48 hours post-transfection, washed twice with PBS, and lysed with 400μl Nonidet P-40 buffer (150mM NaCl, 1% NP40, 50mM Tris [pH 6.8], with complete protease inhibitors (Roche)). Cell debris was spun down and the supernatant quantitated by Bradford assay (Biorad). Cell lysates were incubated with SDS gel loading buffer at 65°C for 5 minutes, and subjected to SDS-polyacrylamide gel electrophoresis. Electrophoresed gels were transferred to nitrocellulose membranes (Hybond, Amersham) by

wet transfer (20V for 16 hours) and blocked in 5% non-fat dry milk (NFDM), 1% Tween-20 Tris Buffered Saline (TBSt) for 30 minutes at room temperature.

Flag-HRP antibody (Bethyl) was used at 1:10,000 dilution in 3% NFDM-TBSt. HA-HRP antibody (Sigma) was used at 1:10,000 dilution in 1% BSA in TBS-T. Goat anti-β-actin antibody (Cell Signaling) was used as a control at 1:1,000 dilution and the secondary peroxidase-conjugated anti-goat antibody (Dako) was diluted to 1:2,000, both in 5% NFDM. Rabbit anti-IKKγ, -IκBα, -NFκB-1, -RelB and -TRAF-6 (Cell Signaling) were used at 1:500 and secondary anti-rabbit peroxidase antibody (Cell Signaling) was diluted to 1:2,000, both in 5% NFDM. Membranes were washed for 30 minutes with TBST and Western blots were incubated in SuperSignal Chemi-luminescent substrate (Pierce).

Immuno-precipitations were performed by pre-clearing protein lysates with 2.5ug normal mouse IgG and 50µl of a 50% AG bead:PBS slurry followed by incubation with in a nutator at 4ºC for 30 minutes. After spinning down pre-cleared lysates, 1µg monoclonal anti-IκBα antibody (Cell Signaling) was added and incubated overnight at as before. Fifty microliters of a 50% AG bead:PBS slurry were added and incubated for another hour before washing with PBS thrice. After final spin down the beads were resuspended in 40µl load buffer and submitted to Western blot analysis as described above.

RESULTS

In order to identify the potential role of Orf64 in NFκB signaling we performed transient transfections of HEK-293 and stable KSHV-infected 293 cells (K293) with empty vector or a full-length clone of KSHV Orf64. Forty-eight hours post-transfection cell lysates were

harvested, pre-cleared and immune-precipitated (IP) with anti-IκBα antibody. The protein-antibody conjugates in the IP reaction were pulled down with AG-beads and submitted to western blot analysis. We chose IκBα since it is poly-ubiquitinated (ref) and therefore a possible target for a DUB. IκBα plays a central role in the activation of the canonical NFκB pathway physically sequestering the nuclear factor in the cytoplasm where it is non-functional. The originating lysate was also loaded as an input control for the expression of Flag-tagged Orf64. As shown in Figure 2, expression of Orf64 causes depletion of IκBα protein from the cell. This phenomenon was observed in both naive 293 and KSHV-infected 293s.

The degradation of a cellular protein by a recently described DUB seemed enigmatic since DUB activity generally correlates with protein stability. Thus, we decided to ask whether Orf64 had an effect on the signaling/regulatory molecules upstream of IκBα. We focused on the canonical pathway since it is induced by pro-inflammatory cytokines and pathogenic stimuli through tumor necrosis factor receptor (TNFR) and toll-like receptor (TLR) activation. HEK293 cells were transiently transfected for ectopic expression of Orf64 or empty vector negative control. Lysates were loaded in replicates and subjected to Western blot analyses.

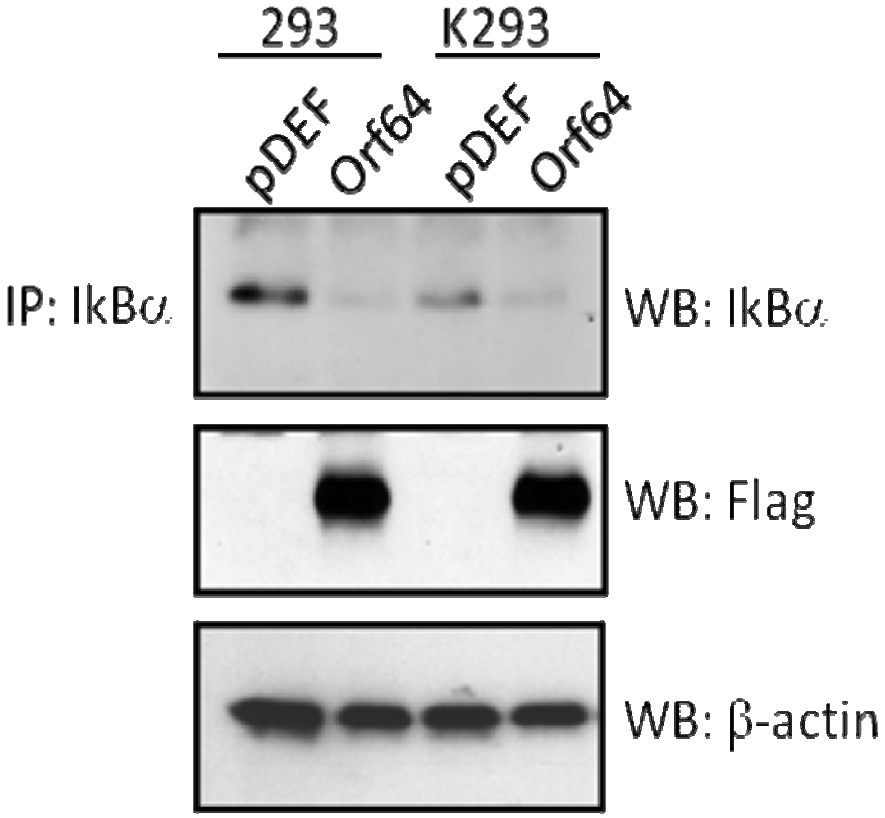

Figure 2. KSHV Orf64 down-regulates IκBα. Expression of KSHV Orf64 results in the degradation of IκBα as shown by IP:WB (upper panel). The middle panel shows Orf64 expression from input. Lower panel shows equal loading of input.

The previously observed IκBα degradation shown in Figure 2 was replicated. Our results, shown in Figure 3, indicate that in the presence of Orf64 the levels of multiple members of the NFκB signaling cascade were reduced. The reduction in total protein levels was marked for IκB and there was a small decrease in IKKγ and not much difference in TRAF-6 levels. TRAF-6 and IKKγ are both modified and indeed regulated through K48- and K63-linked ubiquitination, respectively.

Since IκBα is being degraded, and because Orf64 is a lytic protein, these results led us to ask how the status of NFκB signaling changes during reactivation from latency in our KSHV-293 cell culture system. In order to address this question we plated and twenty-four hour later, induced KSHV-293 cells into lytic reactivation. Cells were incubated for 72 hours post infection to allow ample time for Orf64 expression. Lysates were harvested, total protein concentrations quantitated for equal loading and subjected to Western blot analysis.

The resulting Western blots showed a specific increase in the level of NFκB-2 p100 protein, suggesting that the NFκB-2 subunit is spared from degradation when compared to uninduced control. In contrast, a decreased amount of NFκB-1 p105 protein was present in the induced lane indicating the degradation of p105 and activation of the canonical NFκB pathway (Fig. 4). We also observed a modest decrease in the levels of IKKγ during lytic reactivation. A Western blot for the lytic protein v-IL6 was performed in order to verify the successful induction of the lytic state.

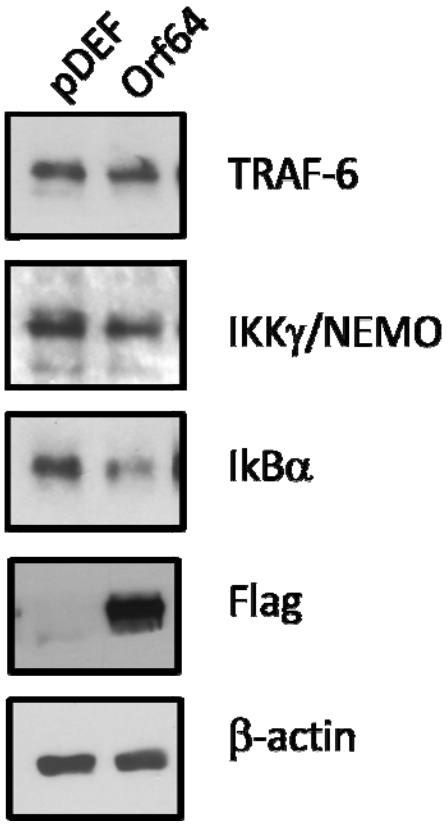

Figure 3. Expression of KSHV Orf64 targets various proteins in the NFκB pathway towards degradation. Ectopic expression of Orf64 can down-modulate levels of IKKγ and IκBα. Second panel from the bottom shows Orf64 expression (Flag), bottom panel show equal loading (β-actin). This suggests that proteins that modulate the stability of IκBα and IKKγ may be targeted by Orf64.

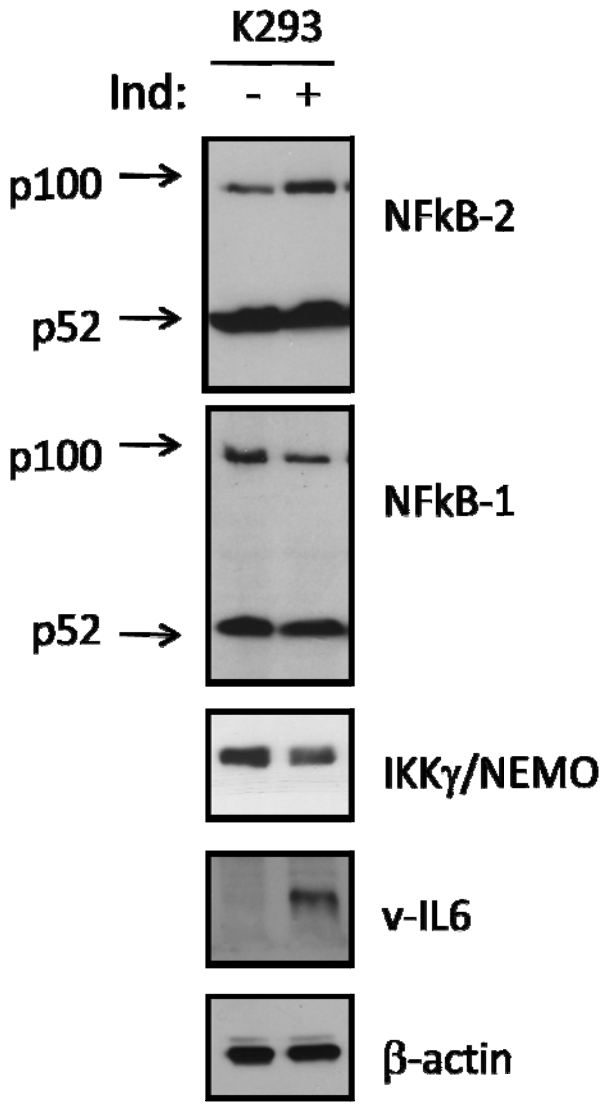

Figure 4. Reactivation from latency triggers the degradation of NFκB-1 p105 and IKKγ and stabilization of NFκB-2. KSHV-293 (K293) cells were induced into lytic replication with baculovirus encoding Orf50 and sodium butyrate or mock-induced. Cell lysates were harvested 72 hours post-induction. Western blots for NFκB-1, NFκB-2, and IKKγ were performed. Antibody to lytic protein v-IL6 was used as a control for lytic reactivation and β-actin for equal loading.

DISCUSSION

In light of the recent reports on the activation state of the NF-κB pathway during viral infection and the fact that several key members in this pathway are subject to ubiquitin regulation, we decided to test whether the Orf64 DUB of KSHV was involved in effective on both lysine 48 (K48) and lysine 63 (K63)-linked Ub chains expanding the list of potential targets of deubiquitination as compared to alpha- and beta-herpesviruses whose DUBs are limited to K48-linked Ub chains.

For our initial approach we chose IκBα, a target of Ub-modification that also represents a junction where several pathway stimuli converge in the canonical pathway of NF-κB activation. The results from the immune-precipitation of IκB from Orf64-expressing cells indicate that Orf64 can somehow induce the degradation of IκB (Fig. 2). This effect would seem to be at odds with its DUB activity which could, in the context of K48-linked Ub chains, result in the stabilization of target proteins. IκBα is in fact ubiquitinated through K48 linkages indicating that Orf64 does not act directly act on IκB but rather on a regulatory protein of IκBα that affects its stability, possibly a molecule working upstream in the signaling pathway.

In order to better understand the effects of Orf64 expression on the NF-κB signaling pathway, we repeated the Orf64 vs empty vector transient transfection of HEK-293 cells. We then performed Western blot analyses on the protein lysates with an array of different antibodies specific for members of the NF-κB signaling pathway that lie upstream of IκBα. The resulting blots confirmed the degradation of IκBα in the presence of Orf64 and

identified IKKγ as an additional target of Orf64-induced degradation (Fig. 3). The degree of protein degradation was evident for IκBα and more modestly for IKKγ.

The possible mechanism of Orf64-dependent decrease in IKKγ levels is intriguing. IKKγ, also termed NEMO, is known as the 'molecular switch' of NFκB activation due to its pivotal role in this pathway. Lysin 63-specific ubiquitination of IKKγ by TRAF-6 is required for activation of the IKK complex, which results in the phosphorylation and subsequent degradation of IκB allowing the p50/p65 heterodimer (or p50 homodimers) to move to the nucleus. TRAF-6 is an adaptor protein which translates stimuli from membrane receptors into IKK complex activation through its E3 ligase activity. This adaptor is a lysine-63 (K63)-specific E3 ligase which can also be ubiquitinated via K48 linkages which allow for its regulation via proteosome-mediated degradation. The K48-ubiquitination of TRAF-6 can be reversed by the cellular DUB A20. A20 inactivates TRAF6 though its DUB activity but has not been implicated in regulating its stability [27]. CYLD is another cellular DUB that can deubiquitinate and thus inactivate TRAF-6 and IKKγ. Thus, Orf64 deubiquitination of IKKγ would have functional effects but should not interfere with its stability.

Several groups have reported the activation of NF-κB signaling during various stages of infection (lytic and latent) [33,34,35,36]. Since Orf64 is expressed in the lytic cycle (see chapter 3, Fig. 7) we decided to test whether induction of lytic reactivation in KSHV-293 cells resulted in activation of this pathway.

KSHV-293 cells were plated and induced with baculovirus encoding the lytic switch RTA protein and incubated for one hour. After incubation the inoculum was replaced with complete media containing 1.7mM sodium butyrate. Seventy-two hours post induction cell

lysates were harvested for Western blot analysis. An antibody to the KSHV lytic protein v-IL6 was used to ensure that the lytic cycle had been induced. We then probed membranes with antibodies to NF-κB-1 and NF-κB-2 in order to verify our data in Fig. 3. As predicted, the NF-κB-2 Western blot did not show an increase in degradation of the p100 subunit, whereas the NF-κB-1 blot showed a decrease in the levels of the p105 subunit. These results confirm that lytic reactivation in latently infected KSHV-293 induces activation of the canonical NF-κB pathway. IKKγ was marginally reduced (Fig. 3) in this context. The degradation of NF-κB -1 p105 subunit is mediated by K48-linked poly-ubiquitination. Upon phosphorylation by the IKK complex, IκBs and p105 are ubiquitinated with K48-linked chains by $SCF^{\beta TrCP}$ resulting in their proteosome-mediated degradation.

One possibility is that Orf64 could be stabilizing the ubiquitin-conjugating enzymes responsible for the degradation of the NFκB p105 subunit. Another possibility is that Orf64 could be inducing the degradation of an inhibitor of p105 processing.

These results argue for an effect of Orf64 on the canonical NF-κB pathway and possibly its activation. Ectopic expression of Orf64 in the absence of the viral genome is sufficient for the degradation of IκBα. The ubiquitin-mediated degradation of this central member of NFκB signaling is required for activation. In addition, an IκBα mutant with ablated IKK phosphorylation sites (IκB super-repressor, IκBSR) has been reported that is refractory to degradation by the proteasome, resulting in blocked NFκB signaling and highlighting the key role if NFκB in this pathway. The immediate effects of IκBα degradation are well understood. However, the result of IKKγ down-modulation by Orf64 is less clear and will require further study.

The identification of signal modulating capabilities encoded by Orf64, adds to the body of functions of this multifunctional protein. There are a number of predicted roles in microtubule transport and delivery of DNA into the nucleus based on sequence homology to other herpesviral DUBs. However, KSHV Orf64 has only been reported to be involved in tegument assembly [5]. We have shown that Orf64 encodes DUB activity capable of cleaving both K48- and K63-linked Ub chains. Moreover, Orf64 can also synergize with RTA (Orf50) to increase transcription of lytic promoters. Lastly, we have shown here that Orf64 can also target IκBα for degradation and may additionally de-stabilize IKKγ, an upstream component of the canonical NFκB pathway (Fig. 4). These studies have identified a number of possible targets of Orf64 including IKKγ and IκBα. It is not yet clear that the DUB function is pivotal in this respect, however, the high degree of ubiquitin-mediated regulation in this pathway make this a likely scenario. Future goals will be to determine if the DUB function of Orf64 plays a role in this pathway.

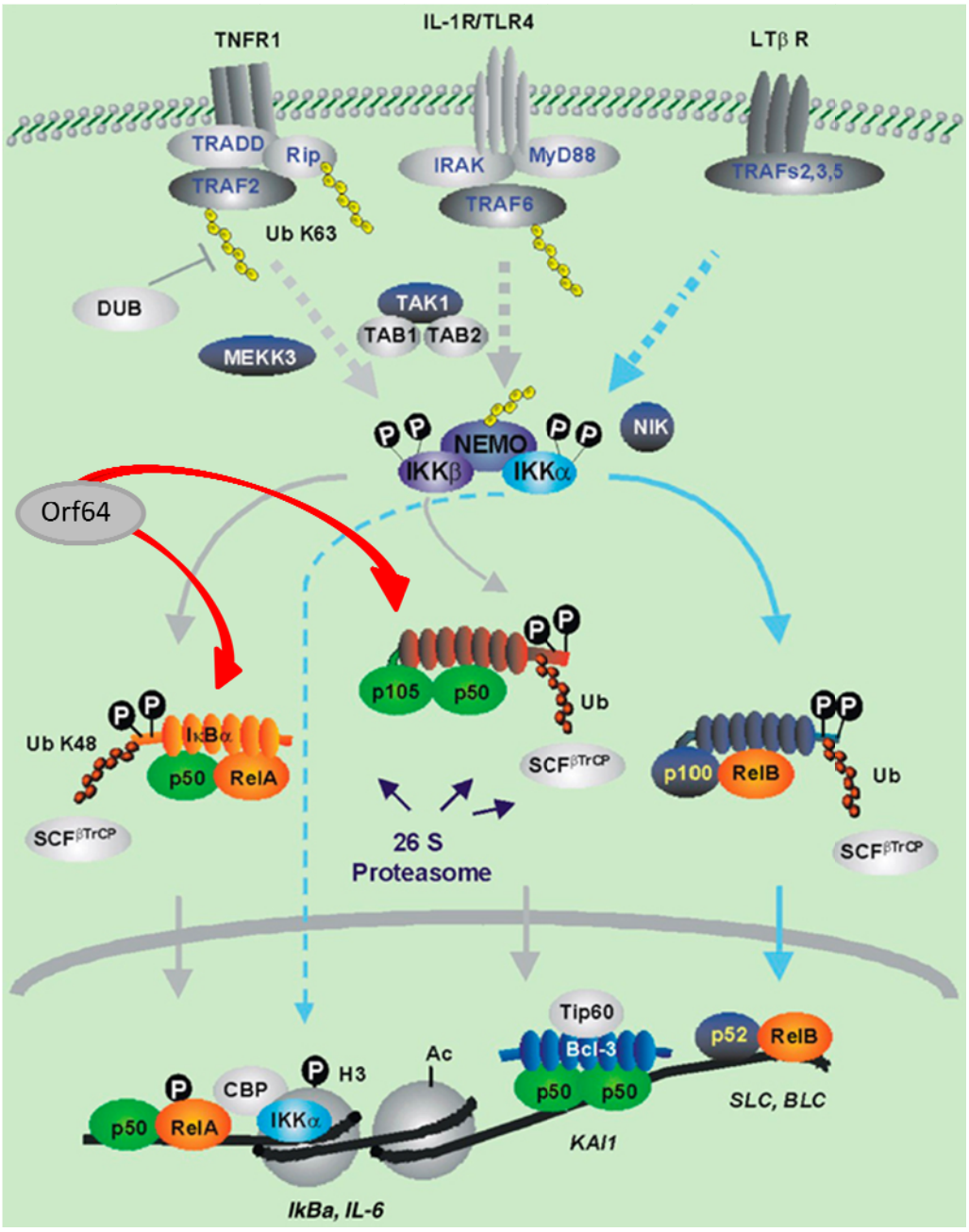

Figure 5. Model of Orf64 mediated activation of the canonical NF-κB pathway. Orf64 expression results in the accelerated degradation of IκBα, and p105 which signal the activation of the NF-κB canonical pathway. (Adapted from Scheidereit, 2006)

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CHAPTER FIVE

GENERAL CONCLUSIONS

General conclusions

Herpesviruses tend to co-speciate with their hosts. This makes infection of other species more difficult but, conversely, translates to a high degree of viral adaptation to the host. This phenomenon is evidenced by the predominantly asymptomatic infection of humans with most of the herpesviruses. A case could be made that a balance has been reached where the virus persists and the host is spared significant viral-induced pathogenesis. This dormant or 'latent' state of infection is a defining feature of herpesviruses. Only sparingly will HSV-1, or HCMV reactivate from latency and cause a short-lived disease episode in the immunocompetent host. Lytic reactivation triggers the activation of the host immune system and controls viral replication by killing infected cells bearing viral antigens.

The most recently discovered human herpesvirus, the Kaposi's Sarcoma-associated Herpesvirus (KSHV or HHV8) (10) is also predominantly latent in the host and it has been identified as the etiological agent of a number of malignancies. KS, PEL and MCD are etiologically associated with KSHV (9, 29)and are most aggressive in the context of the immune-compromised host (17). A primary example is provided by the exponentially increased incidence of KS concordant with the nascent AIDS epidemic in the early 1980s (3). In these cases, the balance between the infectious agent and the host is perturbed by the onset of immune-suppression. The HIV virus targets, replicates in, and kills most of the host T cells resulting in immune-suppression and the development of AIDS. At this time the host immune system is unable to control herpesviral replication and associated pathologies.

Latency is characterized by expression of a very limited set of genes that assist in episome maintenance. The latency associated nuclear antigen (LANA) tethers the viral

episome to the host chromosomes (11). This ensures that the episome is replicated by the host machinery and passed to daughter cells during mitosis. The latent state however, does not last indefinitely. Environmental stimuli like hypoxia can trigger the lytic phase of replication (12). Hypoxia induces expression of the hypoxia-inducible factor 1α (HIF-1α) (33). HIF-1α can bind the promoter for the lytic switch protein encoded by Orf50 and induce its transcription (8). The replication and transcription activator (RTA) encoded by Orf50 is known to be necessary and sufficient for entry into lytic replication (24, 32, 36). The highly-ordered series of events that result in lytic replication begins with immediate early genes like RTA transactivating promoters for early genes. These initial steps require the establishment of self-sustaining positive feedback loops that amplify a starting signal into a cell-wide, coordinated virus production effort. One example of such a feedback loop is the induction of Orf57 by RTA. RTA can induce Orf57 transcription resulting in an increase in Orf57 levels (23). Orf57, in turn, can bind RTA and induce expression from the Orf50 promoter enhancing Orf50 expression and lytic reactivation (26).

This example also illustrates the focus of this dissertation. We have examined additional herpesviral proteins that play important roles supporting RTA in its functions. We have identified two such proteins encoded by KSHV. The first protein we described is encoded by a gene in the lytic switch locus which also contains RTA and K8. The RTA transcript undergoes a splicing event that excludes a sequence of 1kb from the mature RNA (16). It is this sequence in the complementary strand that encodes Orf49. Because of its location in the Orf50 locus we hypothesized that Orf49 could be a previously undescribed member of the lytic cascade. The ends of the Orf49 transcript were identified by 5' and 3'

RACE and revealed it to be unspliced with over 900 bases of coding sequence, a 5' UTR and a polyA tail. As expected, the Orf49 transcript was highly induced early during lytic replication peaking 36hrs post-induction and maintained at high levels for the duration of treatment. Northern blots for Orf49 identified a lytic, 1.2kb transcript that closely matched our full-length cDNA clone.

Transient expression of an Orf49 clone into cells resulted in a protein of ~30 kilodaltons capable of translocating to the nucleus. We showed this by both immuno-fluorescence assays and cell fractionations followed by Western blot. This finding led us to ask whether the nuclear localization of Orf49 was tied to a potential transcriptional activation function. In order to test this hypothesis we performed viral promoter reporter assays. Orf49 proved to be unable to transactivate promoters on its own. However, when combined with Orf50 we observed a synergestic increase in Orf50-mediated transcriptional activation (16). The K8, Orf57 and vGPCR promoters were induced several fold over Orf50-mediated induction levels which ranged from 50-fold for Orf57 to 350-fold for vGPCR over that of empty vector. The K8 or K-bZIP gene product is required for lytic replication since it binds the OriLyt (13). As discussed above, Orf57 transcription is induced by Orf50 and *vice versa* in a positive feedback loop. Thus, an increase in Orf57 transcription would translate into higher levels of Orf50. vGPCR, a constitutively-active G protein-coupled receptor has been implicated in transformation and the angiogenec process via its induction of vascular endothelial growth factor (VEGF) (1, 37). The strong activation of Orf57, vGPCR and K8 promoters suggest that synergy of Orf49 with Orf50 may result in the increased efficiency of lytic reactivation. Because Orf49 was unable to single-handedly induce promoters we hypothesized that it could instead activate a cellular transcription factor. In order to identify

the mechanism of action of the cooperative promoter activation we tested a panel of transcription factor reporter plasmids for activation in the presence of Orf49. We found that the c-Jun transcription factor was activated by Orf49 while ATF1, ATF2 and CREB were not. The induced viral promoters all share AP-1 sites in addition to RTA-responsive elements (RREs) that would respond to c-Jun activation.

Increased phosphorylated c-Jun, JNK and p38 levels were observed upon ectopic expression of Orf49. Orf50 has been shown to increase the levels of total c-Jun in DG75 cells, however it does not induce phosphorylation of c-Jun (34). Together these results suggest a mechanism though which Orf50 increases total c-Jun protein, and Orf49 induces phosphorylation and activation of c-Jun thus increasing c-Jun transcriptional activation potential in the cell.

Cellular stress, growth factors and cytokines all activate JNK and p38 pathways. They share pathway components like the mitogen activated protein kinase kinase kinase (MAP3Ks), and MAP2K (mitogen activated protein kinase kinase) MKK4, and are often co-activated (38). These pathways mediate proliferation, differentiation or apoptosis while p38 activation is also involved in development, and inflammation. KSHV vGPCR expression can also activate JNK and p38 (14). The activation of a signaling pathway by more than one viral Orfs highlights the significance of these pathways to viral replication.

We then set out to assess the involvement of these pathways in lytic reactivation. Pharmacological inhibitors of both JNK and p38 blocked TPA-induced expression of early genes like vGPCR and Orf57 and the late structural gene Orf62/mCP. These data indicate that signaling through both pathways is required for efficient lytic replication.

Taken together our data suggest that Orf49 can induce the activation of the JNK and p38 pathways in order to enhance transcription from key Orf50-responsive promoters. Furthermore, we showed that the activation of the JNK/p38 kinase pathways is essential for KSHV viral replication.

The second gene we have described is that encoded by KSHV Orf64. This Orf is a core herpesviral gene present in all herpesviruses. It is usually the largest ORF of the herpesviral genome and encodes a tegument protein. Various roles of this protein have been reported for HSV-1, PRV and CMV, among them delivery of genomic DNA into the nucleus, egress from the nucleus, and scaffolding during tegument assembly (5, 22, 25). One function shared by all Orf64 homologs studied thus far is deubiquitinase (DUB) or ubiquitin-specific protease (USP) activity. The DUB domain is usually encoded in the N-terminal ~300 residues.

DUB activity affects plaque size, titers, and neuroinvasiveness of PRV with mutations exerting different consequences *in vitro* and *in vivo* (6, 25). The HSV-1 homolog termed UL36, is essential for microtubule transport to and from the nucleus. These data suggest that UL36 homologs play important roles in the lytic replication cycle.

It is intriguing that the actual targets of the DUB activity for most herpesviruses have not been identified. In a recent report, Whitehurst et al (35) described the identification of ribonucleotide reductase as the first known target of the EBV-encoded DUB termed BPLF-1.

In our study of KSHV Orf64 protein, we found that it was present in both nuclear and cytoplasmic compartments. Using *in vitro* deubiquitinase assays, we were able to show that a

GST-Orf64$_N$ fusion protein was capable of cleaving K63- and K48-linked ubiquitin chains. In cell culture, expression of the N-terminus and full-length Orf64 proteins potently reduced the amounts of ubiquitinated proteins, suggesting that Orf64 can deubiquitinate cellular proteins *in vivo* and that this activity is encoded within the N-terminal 200 amino acids.

We then mutated the cysteine 29 residue, a part of the predicted catalytic core of the DUB, in order to functionally identify the source of DUB activity. The resulting N-terminal mutant Orf64 protein (Orf64$_{N\text{-}C29G}$) was devoid of DUB activity both *in vitro* and *in vivo*. From this we conclude that C29 is part of the catalytic core or catalytic triad that defines the cysteine protease active site as predicted by sequence analysis.

KSHV Orf64 localized the nucleus, which is expected given the behavior of its homologs and their roles in virion assembly and DNA packaging. However, given the growing amount of evidence implicating ubiquitination (both destructive and non-destructive) in controlling signaling pathways and transcription factors, we decided to test Orf64 for transcriptional regulatory effects. Herpesviral DUB homologs so far have not been shown to affect transcriptional regulation. We performed promoter reporter assays and found no direct effect of Orf64 on lytic promoters. Although not an effective inducer on its own, Orf64 boosted RTA/Orf50 levels of transcriptional activation several lytic promoters. vGPCR, gB, RTA, and Orf57 promoters were induced more strongly when co-transfected with both RTA and Orf64 *versus* RTA alone.

Upregulation of Glycoprotein B (gB) which helps mediate entry could enhance both the infectivity and amount of virions released (15). As discussed above, the vGPCR protein promotes proliferation and angiogenesis by inducing the release of VEGF into the

microenvironment (2). The RTA and Orf57 proteins can both induce transcription from the RTA promoter (13, 28), suggesting that Orf64 may support lytic replication by inducing RTA expression.

In order to test the significance of Orf64-induced, Orf50 upregulation we knocked-down Orf64 expression by transfection of double stranded short-interfering RNAs (siRNAs). siRNA knockdown of Orf64 in KSHV-infected 293s induced into lytic replication resulted down-regulation of Orf57 lytic transcripts and reduced levels of the lytic protein, vIL6. These data suggest that Orf64 expression can enhance the KSHV lytic cycle, most likely through its induction of lytic gene expression including Orf50. It is unclear at this juncture if deubiquitination activity is required for this particular effect.

The ability of KSHV Orf64 to catalyze cleavage of both K48- and K63-linked ubiquitin sets it apart from all its known alpha- and beta- herpesviral homologs. The upregulation of RTA-mediated lytic promoter activation suggests that Orf64 may play a novel role in enhancing lytic replication, in addition to roles in tegument and virion assembly predicted by homology. The Orf64 protein is contained in the tegument of the incoming virion during *de novo* infection (30) raising the possibility that it may be active upon entry. In this context, Orf64 is poised to modify the cellular environment to favor efficient viral replication before the onset of viral transcription and genome replication.

Recently, reports have emerged implicating ubiquitin as a modulator of the NF-κB pathway. In addition, the activation state of the NF-κB pathway during KSHV infection has been called into question by conflicting reports involving cells of different lineages (18, 21).

Since several key members in the NF-κB pathway are subject to ubiquitin regulation, we asked if Orf64 affects the NF-κB signaling pathway.

We chose the ubiquitination target IκBα as a starting point, convergence point for several pathway signals in the canonical arm of NF-κB pathway activation. During ectopic expression, Orf64-expressing cells showed degradation of IκB. This was contradictory to its DUB activity, which we predicted would result in stabilization of IκBα since it is the subject of K48-linked ubiquitination. This suggests that Orf64 DUB does not act directly on IκBα but instead may stabilize an IκBα regulatory protein operating upstream in the pathway.

We also identified IKKγ or NEMO as an additional target of Orf64-induced degradation. IKKγ is referred to as the 'molecular switch of NFκB activation' because it plays a central role in the pathway. TRAF-6 ubiquitinates IKKγ through K63 linkages, which is required for activation of the IKK complex. Activation results in the phosphorylation and degradation of IκBα, freeing NF-κB dimeric subunits to translocate to the nucleus. The TRAF-6 adaptor molecule is a K63-specific E3 ligase that is also subject to K48-linked ubiquitination and degradation. Ubiquitinated TRAF-6 is a substrate of the cellular DUB A20. A20 deubiquitination of TRAF-6 inhibits its activity (4). TRAF-6 and IKKγ are also substrates of CYLD, a cellular DUB that can deubiquitinate and inactivate both.

NF-κB signaling during lytic and latent stages of infection has been reported (18, 22). Because Orf64 is a lytic protein we asked whether lytic reactivation in KSHV-infected 293 cells resulted in activation of this pathway. Our results indicate that lytic reactivation of latently infected KSHV-293 induces activation of the canonical NF-κB pathway since the

levels of the p105 (NF-κB-1) subunit were decreased. IKKγ levels were also reduced but to a lesser extent. NF-κB-1 p105 subunit degradation is K48-linked poly-ubiquitination dependent. The IKK complex phosphorylates IκBs and p105, which are then K48-ubiquitinated by $SCF^{\beta TrCP}$. This targets both proteins for processing by the proteosome. Orf64 could thus act to stabilize ubiquitin E3 ligases like $SCF^{\beta TrCP}$ which mediate the degradation of the NFκB p105 subunit by K48-mediated deubiquitination of these proteins. Alternatively, Orf64 may interfere with the activation of inhibitors of p105 processing by K48-mediated deubiquitination of these proteins.

Our results suggest that Orf64 plays a role in activation of the canonical NF-κB pathway since expression of Orf64 results in the degradation of IκBα. This particular member of the NFκB pathway lies at a bottle neck where several different stimuli converge on IκBα to be either allowed to proceed or negated. A IκBα mutant with ablated ability to phosphorylate, and thus activate (IκB super-repressor, IκBSR) has been reported to block NFκB signaling (19). This mutant protein highlights the key role of IκBα in NFκB signaling. The Orf64-mediated down-modulation of IKKγ is intriguing and will require further study.

NF-kB is activated during infection of HUVECs and blocking this activation results in cytotoxicity (18). EBV and KSHV positive B-cell lines are mainly latent and also have active NF-κB signaling (7, 20, 21, 39). These cells apoptose upon pharmacological inhibition of NF-κB, suggesting that this pathway is important in maintaining latency. Orf64 is present in the incoming virion during de novo infection and could initiate NF-κB activation before viral gene expression. This could enhance the avoidance of cytotoxicity by the action of pro-

survival products of NF-κB and lytic replication by induction of viral promoters. In fact, KSHV vGPCR is a strong activator of the NF-κB pathway (27, 31) indicating that Orf64 may be able to induce NF-κB signaling by two routes; degradation of IκB and lytic promoter transactivation.

The sum of the data presented here demonstrated that there are viral proteins that support the role of KSHV RTA during lytic replication by different mechanisms. In our studies we found two examples of this phenomenon. First, the early lytic protein encoded by Orf49 was shown to act as an activator of the JNK and p38 signaling pathways. We subsequently showed that activation of these pathways is crucial for effective lytic replication and virion production.

A second example is the activation of the NFκB pathway by Orf64. The exact mechanism for NF-kB activation is not yet clear but it is reproducible. As discussed in chapter 3, proteosomal degradation is not the only outcome of ubiquitination. Functional regulation of proteins is widespread and K63-mediated regulation is of particular importance in the NFκB pathway. Thus, there is a large pool of potential targets of Orf64-mediated deubiquitination. Moreover, since Orf64 is present in the incoming virion it is possible that it could activate NF-κB signaling upon entry. Orf64 could also activate signaling during the late lytic phase where it is expressed (Fig. 1).

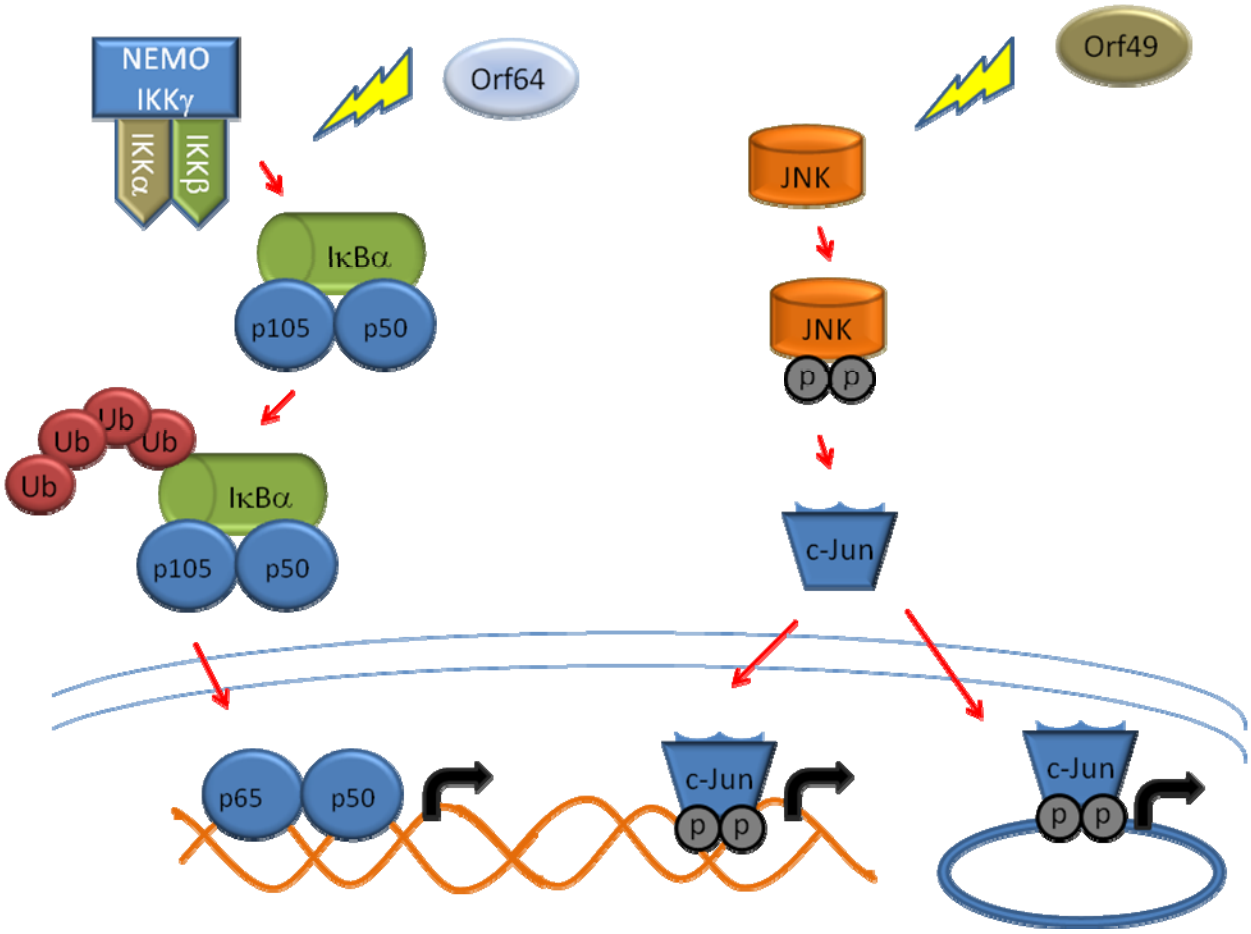

Figure 1. Model of signaling pathway induction by KSHV Orf49 and Orf64. Orf49 activates JNK and the c-Jun transcription factor to induce transcription of both cellular and viral genes. JNK signaling activation is essential for the completion of the lytic cycle. Orf64 targets IκBα and perhaps IKKγ for degradation inducing the activation of the canonical NF-κB pathway.

The work discussed in this thesis documents the contributions of two previously undescribed KSHV proteins to RTA-mediated induction of lytic replication. We believe more examples of viral modulators of the RTA protein will emerge and further delineate the mechanisms through which RTA achieves the efficient induction of the lytic cycle.

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