The First Open-Reading Frames of Kaposi's Sarcoma-associated Herpesvirus and Rhesus Monkey Rhadinovirus and their Contributions to the Viral Life-Cycle

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ABSTRACT

CHRISTINE C. TOMLINSON: The First Open-Reading Frames of Kaposi's Sarcoma-associated Herpesvirus and Rhesus Monkey Rhadinovirus and their Contributions to the Viral Life-Cycle
(Under the direction of Blossom Damania)

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's Disease. The simian homologue of KSHV, rhesus monkey rhadinovirus (RRV), is highly related to KSHV. The first open reading frames of KSHV and RRV encode for proteins K1 and R1, respectively. Although only 17% similar at the amino acid level, K1 and R1 function similarly, scoring positively in a number of cellular transformation assays and encoding an immunoreceptor –tyrosine-based activation motif (ITAM). Both proteins are capable of activating B lymphocyte signal transduction and interacting with the major B cell kinase, Syk.

Expression of K1 in B lymphocytes was found to activate the PI3K/Akt pathway and inhibit PTEN, leading to the inhibition of FKHR transcription factors, key regulators of cell cycle progression and apoptosis. K1 expression inhibited apoptosis induced by FKHR proteins and the FasL/Fas receptor pathway. Expression of K1 promotes cell survival pathways and contributes to KSHV pathogenesis by preventing virally infected cells from undergoing premature apoptosis.

Recent evidence suggests that receptor signaling not only occurs at the cell membrane, but from intracellular compartments. K1 was found to internalize in a clathrin-dependent manner, trafficking from the early endosome to the recycling endosome. By blocking signaling of PI3K or Syk, the rate of K1 internalization was diminished. Additionally, blocking clathrin-mediated endocytosis inhibited downstream signaling by K1 to Akt. The above phenomena are dependent on a functional ITAM of K1. This suggests that K1 signaling is strongly associated with internalization. In B-cells, K1 co-internalizes with the BCR, suggesting that K1 scavenges the BCR from the surface.

In order to discern what role R1 plays in the virus life-cycle, the R1 ORF was deleted from the RRV genome, by insertion of a GFP expression cassette. By PCR and Southern blot analysis, this virus is identical to the wild-type virus, except for insertion of the transgene. This virus will be used to analyze the contribution of R1 to lytic replication, establishment of latency, and reactivation from latency. Additionally, this virus can be used to observe the role R1 plays in the development of disease *in vivo.*

“We don’t receive wisdom; we must discover it for ourselves after a journey that no one can take for us or spare us”.

-Marcel Proust

DEDICATION

To my parents, for their love and support.

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
BAC	Bacterial artificial chromosome
BCBL	Body cavity based lymphoma
BCR	B Cell Receptor
bp	Base pairs of DNA
BJAB	Burkitt lymphoma derived B-cell line
cDNA	Complementary DNA
CMV	Cytomegalovirus
CPE	Cytopathic effect
Ct	Cycle threshold
CTL	Cytotoxic T-lymphocyte
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Early class of transcription
EBV	Epstein-Barr Virus
EC	Endothelial cells
EGFP	Enhanced green fluorescence protein
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunoadsorbant Assay
ER	Endoplasmic Reticulum

FLICE	FADD-like IL-1β–converting enzyme
FLIP	FLICE inhibitory protein
FOXO	Forkhead box O-class
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gB	Glycoprotein B
GFP	Green fluorescence protein
GPCR	G protein-coupled receptor
GSK	Glycogen Synthase Kinase
HEK-293	Human endothelial kidney – 293 cells
HHV-8	Human herpesvirus 8 (or KSHV)
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
HUVEC	Human umbilical vein endothelial cells
HVS	Herpesvirus saimiri
kDa	Kilodalton
IE	Immediate early class of transcription
Ig	Immunoglobulin
IGFP	Insulin-like growth factor protein
IL	Interleukin
IP	Immunoprecipitation
IRF	Interferon regulatory factor homologue

IRS	Insulin response sequence
ITAM	Immunoreceptor tyrosine-based activation motif
KDa	Kilo-Daltons of protein
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late class of transcription
LANA	Latency-associated nuclear antigen
MCD	Multicentric Casteman's disease
MHV-68	Murine gammaherpesvirus-68
mRNA	Messenger RNA
MOI	Multiplicity of infection
NERPRC	New england regional primate research center
nt	Nucleotide of DNA or RNA
ORF	Open reading frame
ORPRC	Oregon regional primate research center
PB	Polybrene
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PFA	Paraformaldehyde
PFU	Plaque forming unit
PI3K	Phosphotidylinositol-3 Kinase

Rb	Retinoblastoma protein
RFHV	Rhesus retroperitoneal fibromatosis-associated herpesvirus
RFP	Red fluorescence protein
RhF	Rhesus fibroblast cell line
RIPA	Radioimmunoprecipitation Assay
R-LANA	RRV latency-associated nuclear antigen
RNA	Ribonucleic acid
RRE	Rta responsive element
RRV	Rhesus monkey rhadinovirus
RT-PCR	Reverse transcriptase PCR
RTqPCR	Real-time quantitative PCR
Rta	Replication and transcription activator protein
SAIDS	Simian AIDS
SCID	Severe combined immune deficient
SDS	Sodium dodecyl sulfate
SEAP	Secreted-engineered alkaline phosphatase
SH2	Src-homology-2
TBS	Tris Buffered Saline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electorphoresis
SIV	Simian immunodeficiency virus
TPA	12-*O*-tetradecanoylphorbol-13-acetate
VEGF	Vascular endothelial growth factor
WT	Wild type

CHAPTER ONE

INTRODUCTION

Kaposi's Sarcoma

In 1872, a Hungarian dermatologist described skin lesions that he observed in five elderly male patients, which he initially named "idiopathic multiple pigmented sarcoma" (117). Dr. Moritz Kaposi summarized the disease as follows: "Nodules the size of peas or hazelnuts, brown-red to blue-red in color. 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. They usually appear on the feet, then on the hands. As the disease progresses, isolated nodules and groups of nodules appear on the arms, legs, face and trunk. Finally, identical nodules appear on the mucous linings of the larynx, trachea, stomach, intestines and liver. The disease is rapidly lethal, with death within two or three years" (80, 81). This disease was later named Kaposi's sarcoma (KS).

It is now known that KS is a spindle-cell tumor of endothelial cell lineage. KS has a variable clinical course ranging from minimal mucocutaneous disease to extensive organ involvement. KS has been classified into four clinical groups:

Classic KS: Typically occurs in elderly men of Mediterranean and Eastern European background. Classic KS usually has a protracted and indolent course. Common complications include venous stasis and lymphedema. As many as 30% of patients with classic KS subsequently may develop a second malignancy (54).

Epidemic AIDS-related KS: Occurs in patients with advanced HIV infection, and is the most common presentation of KS. In the United States, KS serves as an AIDS-defining illness in 10-15% of HIV-infected homosexual men. In Africa and developing regions, epidemic AIDS-related KS is common in heterosexual adults and occurs less often in

children. AIDS-related KS is the most clinically aggressive form of KS and remains the most commonly diagnosed AIDS-associated cancer in the United States (19).

Iatrogenic KS: Characteristically occurs following solid-organ transplantation or in patients receiving immunosuppressive therapy. However, individuals with congenital immunodeficient states are not at increased risk for developing KS. The average time to development of KS following transplantation is 30 months (122).

Endemic African KS: Occurs in younger African men who are HIV seronegative and is most typical in sub-Saharan Africa. Striking regional variations in frequency are seen with most cases in the central African region (155). The endemic subtype occurs most frequently in young children and can present with prominent lymph-node involvement (150). Adults with endemic KS can present with typical skin nodules and plaques, which usually cover extensive areas of skin.

Kaposi's Sarcoma-Associated Herpesvirus

Epidemiological evidence suggested that KS, particularly epidemic KS, was being transmitted by an infectious agent (7). Over 20 agents have been suspected to be involved in KS development, including cytomegalovirus (CMV) and HIV. In 1994, Chang and Moore first described the finding of herpesvirus-like genomic sequences in KS lesions from AIDS patients. Using representational difference analysis, they were able to isolate two small fragments from a novel viral genome (39). They termed this virus Kaposi's Sarcoma-Associated Herpesvirus (KSHV). Although these fragments represented less than 1% of the entire viral genome, within two years the entire virus had been sequenced (140).

KSHV-related diseases

Since the initial discovery of KSHV, also called human herpesvirus 8 (HHV-8), it has been found that all forms of epidemiological KS lesions, regardless of their source or clinical subtype, are infected with KSHV (25). Along with KS, KSHV is associated with several other proliferative diseases. These include primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD).

Primary Effusion Lymphoma

PEL, also known as body-cavity based lymphoma, is a diffuse-large B-cell lymphoma, and occurs as malignant effusions in visceral cavities (pleural, peritoneal and pericardial), usually without any visible mass. It appears that most PELs are also infected with Epstein-Barr virus (EBV), although dual infection is not a requirement (36). Majority of afflicted patients are homosexual men who are HIV positive. Once PEL develops, most patients are resistant to conventional chemotherapy and die of their disease within months (36). Cell lines established from PEL, unlike KS tumor explants, stably maintain viral episomes at high copy number (50–150 copies per cell) and are the source of virus for most virologic and serologic studies.

Multicentric Castleman's Disease

Castleman's disease is a rare atypical lymphoproliferative disorder classified according to the histopathologic findings of the affected lymph nodes as hyaline–vascular, plasma-cell type, or a mixed type. MCD is a systemic form of the plasma cell variant with patients exhibiting generalized peripheral lymphadenopathy, hepatosplenomegaly, frequent fevers, and night sweats (123). Only a subset of plasmablasts in MCD tumors are KSHV-infected and the proportion of lytically infected cells is higher than other KSHV-related

diseases (83). Dysregulated IL-6 production is what leads to the characteristic clinical and histologic manifestations (30). KSHV encodes a homologue of cellular IL-6, known as viral-IL-6 (vIL-6). Expression of vIL-6 is upregulated in KSHV-associated MCD, and has been shown to correlate with disease progression (118). KSHV-related MCD is clinically severe, with most patients dying within 2 years (118).

The Herpesvirus Life-Cycle

The *Herpesviridae* family is a large group of viruses, which contain double-stranded DNA genomes. Biological characteristics, such as clinical manifestation, site of replication and site of latency have been used to classify three major subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* within the family *Herpesviridae* (Figure 1) (47). The family name is taken from the Greek verb, *herpein*, meaning "to creep." The name refers to the fact that the members of this family often cause latent, recurring infections which progress slowly (137). The archetypal herpesvirus, Herpes simplex virus type 1 (HSV-1) is a member of the alpha herpesviruses. HSV-1 establishes a life-long latent infection in neural root ganglia and reactivates in innervated epithelial cells, typically of the oral mucosa. This subfamily also includes HSV-2 and Varicella-Zoster virus, the causative agent of chicken pox and shingles (137). The Beta herpes subfamily includes Human cytomegalovirus (HCMV), HHV-6 and HHV-7. HCMV has a long replicative cycle and grows very slowly in culture. Members of the beta family have a very restricted host range and can cause disease in organ transplant patients and young children. KSHV is a member of the gamma-herpesvirus subfamily. The gamma herpesviruses are further divided into two

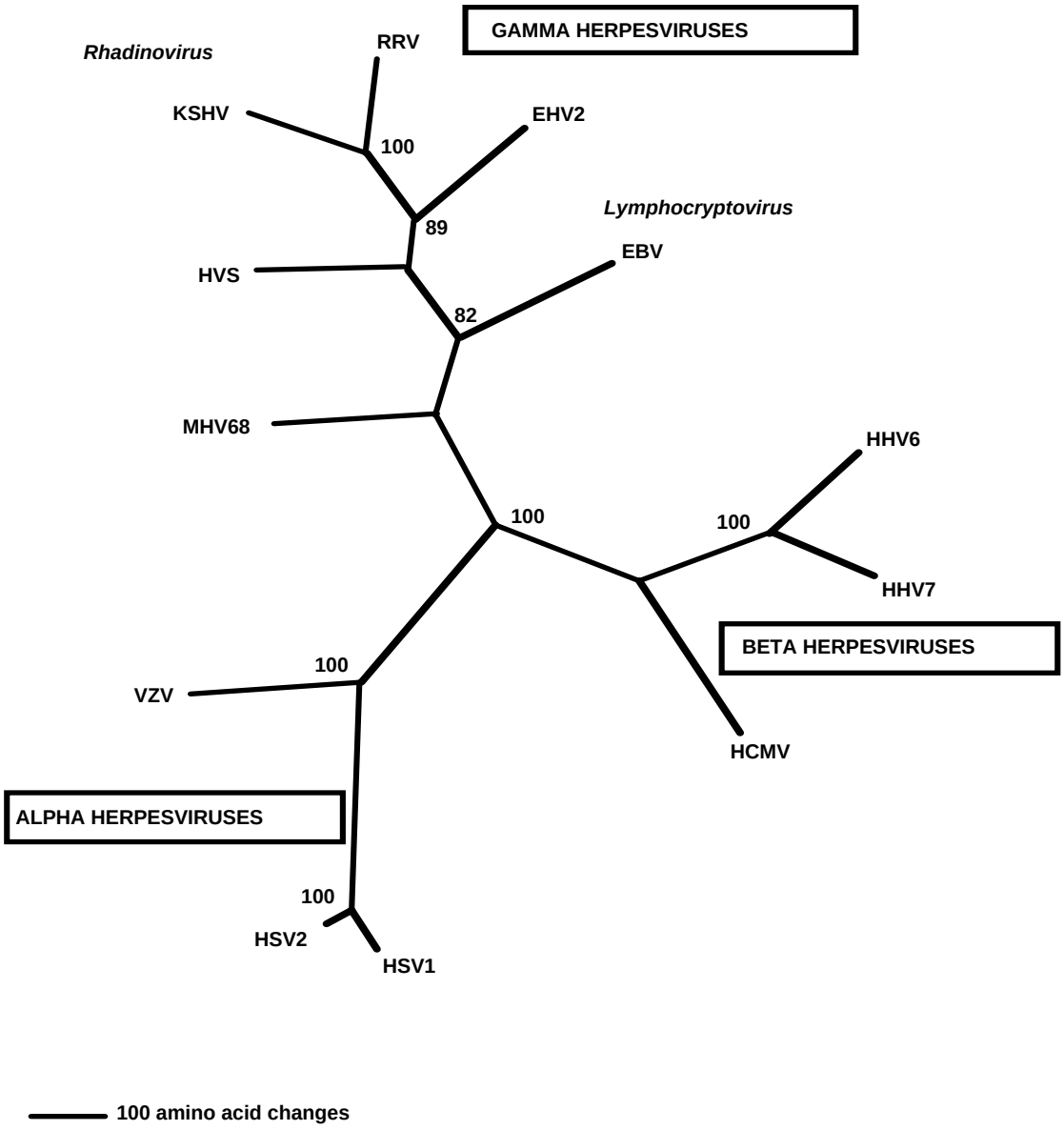

Figure 1. Phylogenetic tree depicting the Herpesvirus Family. This is an unrooted phylogram based on the sequence of the viral DNA polymerase gene by parsimony analysis using the neighbor-joining method. The scale of the number of amino acid changes is shown on the lower-left. (Taken with permission from Damania and Jung (47).)

classes: gamma-1 or lymphocryptovirus, which includes EBV, and gamma-2 or rhadinovirus, to which KSHV belongs. Viruses in the gamma herpesvirus subfamily typically infect T or B lymphocytes and are tightly associated with neoplastic disease. This group of viruses has more sequence similarity to each other than to any other members of the herpesviridae family

Herpesvirus infection of host cells begins with attachment of the virus to the cellular surface receptors via glycoproteins located on the envelope of the virion. Although the cellular receptors and glycoproteins involved for each virus is unique, the overall order of infection and replication is very similar across the subfamilies. For KSHV, the virus initially binds heparin sulfate via gB and K8.1A glycoprotein. gB then binds the host cell surface α3β1 integrin, and utilizes α3β1 integrin as one of the cellular receptors for entry into the human endothelial and fibroblast target cells (1). A recent study has also shown that KSHV utilizes the transporter protein xCT for entry into adherent cells, but not to B cells (79). Once internalized, the virus uncoats and linear viral DNA circularizes as it is transported to the nucleus. Transcription of viral genes, replication of viral DNA and assembly of new capsids take place in the nucleus.

Viral Replication

All herpesviruses have two classes of replication: lytic and latent. During lytic replication, viral DNA is replicated by a viral-encoded polymerase and encapsidated into infectious virions. In the course of latent replication, the viral DNA is maintained in an episomal state, allowing for it to be replicated in tandem with host chromosomal DNA, using host cell replication machinery. Lytic replication is a highly ordered transcriptional cascade in which a viral transactivator gene acts as a master regulator to turn on other

genes. For KSHV, that master gene is encoded by open reading frame 50 (ORF50), known as the replication and transcription activator (RTA) protein. RTA binds directly to a 16-bp core sequence found in many KSHV promoters, known as the RTA responsive element (RRE) (31).

KSHV Lytic Replication

Transcription typically occurs in three waves, termed immediate early (IE), early (E) and late (L). This ordered cascade is necessary, due to the large number of ORFs encoded by KSHV. Co-ordination of gene transcription ensures that specific proteins are available for each stage of replication. IE transcription begins with just a few genes that then transactivate other viral promoters. E genes are typically enzymes that are involved in replication of viral genes, such as DNA polymerase, primase and helicase. KSHV DNA replication is also highly ordered, initiating from a lytic origin (*ori-Lyt*) within the viral genome. Two functional *ori-Lyt*s have been identified in the KSHV genome. Six core replication proteins and two regulatory proteins, namely, RTA and K8, are necessary and sufficient for KSHV *ori-Lyt*-dependent DNA replication (9). RTA binds to a consensus RRE in *ori-Lyt*, and K8 associates with the *ori-Lyt* DNA through interaction with C/EBP α molecules bound on a cluster of C/EBP binding motifs (9). Replication of viral DNA and lytic replication are tightly coupled via the role of RTA. As the master transactivator of KSHV lytic gene expression, RTA is also required for replication of the viral genome (163).

L gene expression begins after DNA replication has begun. Proper KSHV late gene expression is dependent upon virus lytic replication in *cis* (38). Genes expressed in the L phase of transcription consist of viral structural genes and those involved in the

assembly of viral capsids (115). Finally, the tegument and envelope are acquired as the virion buds out through the nuclear membrane or endoplasmic reticulum. Virions are transported to the cell membrane via the Golgi complex, and the host cell dies as mature virions are released.

KSHV Latency

Only a subset of genes are transcribed during latency. For KSHV, these genes include the latency-associated nuclear antigen (LANA) locus that directs the transcription of three latent genes from one promoter, coding for LANA, a viral cyclin (vCyclin), and a viral FLICE inhibitory protein (vFLIP) (55, 131). Other latent genes include the kaposin locus (141), the K15 gene (148) and in some cell-types one of the viral interferon regulatory factor homologues (vIRF) (136). The KSHV microRNAs are also expressed during latency (33). The restriction of gene expression during latency contributes to the ability of the virus to escape immune surveillance and establish a persistent infection (28, 29). The few latent KSHV proteins expressed deregulate various cellular pathways in order to increase the proliferation and survival of infected cells. LANA, encoded by ORF73, is perhaps the most important protein found in latently infected cells. LANA has been shown to have multiple functions, one of which is to physically tether the viral genome to the host chromosome (13). This ensures that the viral genome will be replicated and segregated to daughter cells during mitosis (86, 125, 149). In addition, LANA associates with various cellular proteins including p53, pRb, and GSK3β, stimulating pathways involved in cell survival and proliferation and contributing to the oncogenic process in KSHV-associated malignancies (67, 69, 130, 132).

Reactivation from Latency

Although latency allows KSHV to persist within an infected cell, lytic reactivation of the virus is critical to ensure the propagation of the virus. Additionally, expression of viral cytokine homologues function as paracrine factors in stimulating cell growth and proliferation (11, 12, 37). Impaired cellular immunity plays a critical role in allowing KSHV replication, but the physiological stimuli that induce lytic gene expression from a latent state are poorly understood. Chemical agents such as phorbol esters or *N*-butyrate can reactivate KSHV *in vitro* (100, 131, 143), and proinflammatory cytokines have similar, though weaker effects (29, 99, 110). It has also been shown that hypoxic conditions can reactivate KSHV by stimulating the transcription of ORF50 (32, 49, 72). It is possible that hypoxia is a major contributor to KSHV reactivation *in vivo.* Classic KS, which appears in the apparent absence of immunosuppression, occurs predominantly on the lower extremities, a common site of poor circulation in the elderly. Additionally, many solid tumors have areas of hypoxia; this could contribute to lytic reactivation in a sub-set of infected cells. There is also preliminary data suggesting that some environmental factors may contribute to viral reactivation. The "oncoweed" hypothesis suggests that natural products may contribute to increased reactivation, enhancing transmission of KSHV in KS endemic areas and accelerating progression to KS disease in individuals latently infected with KSHV (164).

The KSHV genome

The KSHV genome is approximately 140-150 kilobases. Like all herpesviruses, it is a double-stranded DNA molecule that replicates in the nucleus as a closed circular episome during latency but is linear during virion packaging and replication. The

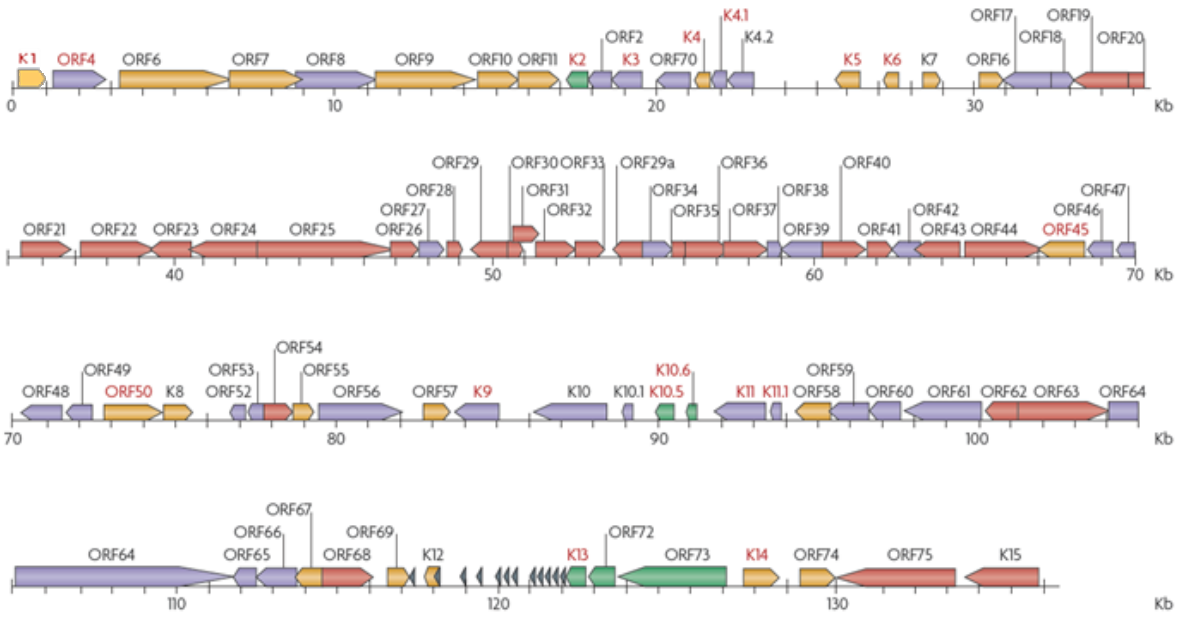

Figure 2. KSHV genome map. Viral genes transcribed early upon reactivation are indicated in yellow, those transcribed during intermediate kinetics in orange, and those transcribed during late kinetics in red. Latently expressed genes are in green. Genes that encode proteins involved in immune evasion are indicated in red font. (Adapted with permission from Coscoy, 2007 (44).)

genome is composed of a unique-long region, flanked by the 20-35 kb terminal repeat region composed of 801-base pair, high G+C content, terminal repeat units (140). Over 80 ORFs have been identified in the unique long region (Figure 2), although not all have an identified function to date. Structural genes and highly conserved genes involved in lytic replication tend to cluster in regions centrally located in the genome. Genes located more centrally tend to undergo much less recombination, whereas the genes located towards the ends show much more sequence variability. In fact, one of these terminal proteins, K1, has been used to define KSHV isolates into subgroups, detailed below.

Like other gammaherpesviruses, KSHV encodes many ORFs that function to modify the host environment to its advantage. Some of these ORFs have homology to other gammaherpesviruses, while some are unique to KSHV and others have homology to cellular genes. Whether this is due to mimicry or piracy of cellular genes is not known. Either way, KSHV has modified these genes to its advantage. Expression of these different proteins results in down modulation of the immune response, disruption of the cell cycle, changes in nucleotide biosynthesis and subversion of cellular signaling and proliferation pathways. KSHV encodes homologues of human cyclin D (vCYC) (98), FLICE inhibitory protein (vFLIP) (16), G protein-coupled receptor (vGPCR) (156), chemokine homologs (vMIPs) (106), homologue of IL-6 (112), proteins with similarity to interferon (IFN) regulatory factors (vIRFs) (111) and a bcl-2 homologue (40).

The K1 Open Reading Frame of KSHV

In addition to cellular homologues, KSHV also encodes several genes that are unique to the virus. Among these genes is K1, located at the far left end of the KSHV genome, immediately adjacent to the terminal repeats. Identified in 1997, K1 was

predicted to have 289 amino acids and function as a Type I transmembrane protein (90). K1 has since been shown to be 46 kiloDaltons, with a predicted signal peptide sequence at the amino terminus, an extracellular domain, a transmembrane domain, and a short cytoplasmic tail at the carboxyl terminus (Figure 3) (127, 171). Although the sequence of K1 from different KSHV isolates is highly variable, there are domains that are highly conserved (96, 171). This includes several glycosylation sites and cysteine residues located in the extracellular domain, and two Src-homology-2 (SH2) binding motifs in the cytoplasmic tail of K1. These two SH2 binding motifs have been shown to constitute a functional immune-receptor tyrosine-based activation motif (ITAM) in that they and the surrounding sequences are spaced in a fashion consistent with that of the ITAM consensus sequence, $(D/E)X_7(D/E)X_2YX_2LX_{7-10}YX_2L/I$. Studies have shown that the ITAM of K1 can induce nuclear factor activated T-cells (NFAT), nuclear factor-κB (NF-κB), calcium mobilization, and phosphorylation of cellular tyrosine residues, all of which are indicative of lymphocyte activation (91, 95, 142) (Figure 3). ITAMs are also found in other signaling molecules associated with immune cells, such as immunoglobulin α and β (Igα, β), CD3γ, FcεRIγ, and several viral proteins such as gp30 of bovine leukemia virus, LMP2A of EBV and Nef of SIV (14, 34, 59, 64, 108, 109). Unlike ITAM-based signaling of cellular proteins, K1 signaling occurs constitutively, mediated by oligomerization via conserved disulfide bonds in the extracellular domains (91, 95, 96). K1 has been shown to be expressed in KS lesions, primary effusion lymphoma cells, and multicentric Castleman's disease (26, 90, 93, 142). Thought to be an early gene, K1 is predominantly expressed during lytic infection and its transcription is upregulated following TPA treatment and is resistant to phosphonoformic acid (PFA), an inhibitor of viral DNA

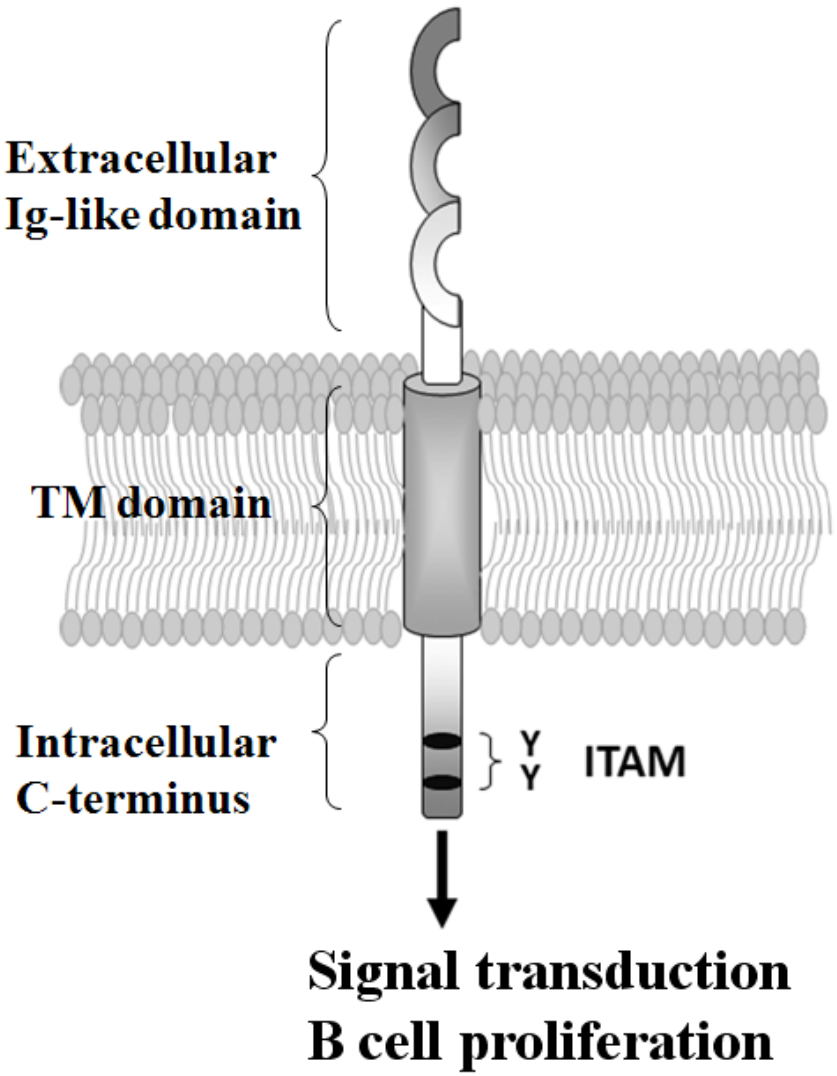

Figure 3.The KSHV K1 signaling protein. A schematic diagram of the KSHV K1 protein. The K1 protein has an extracellular domain, transmembrane domain, and a cytoplasmic tail. The cytoplasmic tail contains an immunoreceptor tyrosine-based activation motif (ITAM). K1 signaling induces activity of NF-κB and NFAT and results in B-lymphocyte activation.

synthesis (90). Analysis of KSHV's transcriptional program has indicated that K1 expression is upregulated during the lytic cycle (78, 84, 121, 144) However, it has been shown that K1 expression is not limited to the lytic cycle. K1 transcripts have been detected at low levels in latently infected PEL cells (62, 90, 142). Recently, Verma *et al* have shown that LANA binds to the K1 promoter, limiting K1 expression during latency. RTA reverses the LANA-mediated repression of the K1 promoter, resulting in K1 upregulation during the latent/lytic switch. Signaling by K1 contributes to the enhancement of lytic replication (158).

K1 has been shown to interact with multiple cellular proteins containing SH2 domains, including Lyn, Syk, p85, PLCγ2, RasGAP, Vav and Grb2 through the phosphorylated SH2 binding motifs that constitute the ITAM (94). Furthermore, K1 expression has also been shown to promote the production and secretion of vascular endothelial growth factor (VEGF) in both epithelial and endothelial cells and to increase matrix metalloproteinase-9 (MMP-9) expression in endothelial cells. These properties of K1 are dependent on the SH2 binding motifs in the K1 cytoplasmic tail (162). Transgenic K1 mice develop tumors with features similar to spindle-cell sarcomas and malignant plasmablastic lymphoma. Moreover, lymphocytes isolated from these transgenic mice show constitutive activation of NF-κB and Oct-2, and enhanced Lyn activity (128, 129).

K1 has also been shown to modulate the expression of the B-cell receptor (BCR). The amino-terminal domain of K1 interacts with the μ chain of the BCR-complex, inhibiting the intracellular transport of BCR by retaining BCR-complexes in the endoplasmic reticulum. This results in decreased surface expression of BCR (92).

KSHV epidemiology

The exact mode of transmission of KSHV is not completely understood and appears to be quite complex. Early hypotheses were that transmission was through a sexual route. One study found that men with homosexual behaviors showed a 38% prevalence of KSHV as compared to 0% of men with no such activity. The increased prevalence correlated with the presence of sexually transmitted diseases (STD) and the number of male sexual partners. Co-infection of HIV and KSHV predicted a 50% probability for developing KS within 10 years (105). However, transmission from male genital secretions, specifically semen, was found to be unlikely due to the low prevalence of detectable KSHV in semen samples obtained from either HIV+ or HIV- persons (76). In 1997, Vieira *et al* found that KSHV was transmitted in salivary secretions (159). Similarly Blackbourn *et al* found that KSHV was present in both salivary and nasal secretions (23). In 2000, Pauk *et al.* also reported that KSHV DNA was found most frequently and with increased viral burdens in saliva (120). Sexual practices that include oral sex could therefore increase the possibility of transmission. That being said, transmission between heterosexual couples is fairly infrequent (151). This is thought to be due mainly to the poor infectivity of KSHV (27, 71), which results in less than 10% seropositivity in the general population outside of KS endemic areas (10). Also contributing to the low-levels of KSHV seropositivity are genetic susceptibility (3, 126) and environmental risk factors (164).

In KS endemic areas, the peak age of acquisition is 6-10 years, which suggests that the length of exposure, as well as repeated exposure are factors that contribute to sero-conversion. Evidence from many studies suggest that KSHV transmission occurs

from mother to child, but this route is horizontal, through saliva, rather than vertical (101, 104). A high level of KSHV DNA in the mother's saliva is the most significant risk for a child to contract KSHV (50, 102).

Studies based on immunofluorescence, Western blot and enzyme-linked immunosorbent assays to detect antibodies against latent and lytic genes, have demonstrated that KSHV is not ubiquitous throughout the general population as are the majority of other human gammaherpesvirus, including EBV. The seroprevalence of KSHV tends to be lowest in Asia, with 0.2% of blood donors in Japan testing positive, compared to 1–3% in the USA. However, this percentage is significantly higher (28%) in certain regions of Italy (68, 135). In areas highly endemic to KS, the range of seroprevalence of KSHV has been reported in some cases to be greater than 50% (146).

The K1 gene of KSHV has been used to identify different clades and sub-groups of KSHV. K1 is located at the extreme left-end of the viral genome. This gene has been shown to have as much as 40% amino-acid sequence variability, resulting from a nucleotide substitution rate of up to 85% (73). The high variability has been suggested to be due to either recombination between different viral clades or due to selective pressure by the host immune-system. It has been found that within K1 there are epitopes for HLA class I-restricted T cells (CTLs), suggesting that CTL-driven evolution has contributed to the sequence variability of K1 (154).

Based upon sequences of K1 from isolates, KSHV was originally designated into four subgroups, A, B, C and D, with 13 different clades (171). B subtypes were found almost exclusively in KS patients from Africa, whereas the rare D subtypes were found only in KS patients of Pacific Island heritage. C subtypes were found predominantly in

classic KS and in iatrogenic and AIDS KS in the Middle East and Asia, whereas U.S. AIDS KS samples were primarily A1, A4, and C3 variants. More recent studies have further divided the virus into six subtypes: A, B, C, D, E, and Z, with more than 24 clades. These data further support a strong association between the KSHV subtype and the geographic origin of the infected host. Subtypes A and C are prevalent in Europe, the U.S.A., and northern Asia. Subtypes B and A5 predominate in Africa and the D variant is found in the Pacific. Subtype E has been discovered in Brazilian Amerindians and a unique subtype Z was found in Zambia (58). A gene encoded at the far right hand side of KSHV, K15, has also been used to genotype viral strains. It has been found that there are two distinct alleles for K15, M and P, which also show geographic distribution (127). Whether a particular viral genotype can be associated with the development or progression of KS (or any of the other KSHV-associated diseases) or a particular epidemiologic form of KS has not yet been determined. Genotyping of numerous, geographically distinct AIDS populations has shown no clear correlation between a particular K1 or K15 variant and pathogenesis of KS, PEL, or MCD (73, 77, 88, 170).

Model systems of KSHV infection

There are a number of KSHV *in vitro* tissue culture models that have been developed thus far. The first such models were derived from patient PEL cells. These B-cell lines harbor KSHV as an episome in 100% of cells even after extensive passage in tissue culture (8, 24, 35, 70, 74, 82). About half of these cell lines also harbor the EBV genome. In PEL cells, the KSHV genome remains predominantly latent, but lytic replication can be induced by chemical induction or ectopic expression of ORF50 (99, 134). However, induced reactivation is not complete. Only about 20% of the cells

undergo lytic reactivation, making the study of the KSHV lytic cycle difficult, as there is an 80% background of latency to contend with. While the majority of PEL cells are latent, there is a background of ~5% spontaneous lytic reactivation, making the study of true latency difficult. PEL lines can also not help us elucidate the mechanisms of how KSHV induces transformation because transformation precedes the establishment of the cell line *in vitro.* Primary B-cells and some established B-cell lines have been shown to be infected by KSHV *in vitro,* but infection does not lead to prolonged maintenance of the viral genome or transformation (15, 22, 116). This is in contrast to endothelial cells which have been shown to be transformed by KSHV infection *in vitro* (6, 65).

Many labs have strived to define model systems in which to study KSHV lytic replication and *de novo* infection. Endothelial and epithelial cells have been shown to be targets of lytic replication *in vivo.* In KS lesions, spindle cells harbor the KSHV genome, but all cell lines developed from KS lesions rapidly lose the viral genome after serial passage *in vitro* (4, 5, 17, 97, 152). Endothelial cells (ECs) are thought to be the precursors of spindle cells in KS. Many EC-based *in vitro* systems have been described and differ slightly, with respect to parental tissue and manner of immortalization, but all have yielded valuable information regarding KSHV biology and pathogenesis. These include the induction of KS markers, a spindle cell phenotype, and transformation (6, 65). Both primary EC (43, 65, 75, 84, 127, 160) and immortalized EC (87, 89, 113, 157) infection models have been developed. However, the majority of these systems fail to support sustained lytic propagation of KSHV, and latency becomes the predominant state of infection. Eventually, most of these cells lose the KSHV episome. More recent studies suggest that infection of more specific EC lineage, particularly lymphatic ECs, results in

long-term maintenance of the viral episome and may be a more relevant model for future studies (160).

Much of the studies of KSHV have entailed the individual cloning and characterization of viral gene products without viral infection. This type of analysis has proved useful in defining mechanisms of individual gene products of KSHV, but does not shed light on the particular gene as it relates to other viral gene products and to the virus itself. This has changed with the recent cloning of the KSHV genome into a bacterial artificial chromosome (BAC) (168). This system allows for the manipulation of the viral genome, either individual genes or multiple genes and attainment of pure recombinant viruses. A number of recombinant viruses have been made with this system (85, 166, 167, 169). However, the limitations of *in vitro* culture systems described above still apply, so examination of properties of the recombinant viruses is still limited. Therefore, the creation of applicable *in vivo* model systems is crucial.

The majority of animal model systems for KSHV have focused on over-expression of single viral oncogenes in transgenic systems including, K13 (42), LANA (63) and K1 (129) or the *in vivo* growth of malignant cell lines, for which it is difficult to evaluate *de novo* viral tropism and gene expression . These studies have used xenografts of BCBL-1 cells in NOD/SCID mice (45, 124) BCP-1 cells in NOD/SCID mice (24), and PEL cells embedded in matrigel injected into C.B.17 SCID mice (153).

There have also been studies that have described KSHV gene expression within human cells, including a xenotransplantation model optimized for the study of HIV-1 infection (41), another that supported propagation of human PBMC (56), and a third that employed direct viral infection of implanted human skin (66).

One of the most recent studies evaluated long-term KSHV infection within an immunocompromised animal host. Parsons *et al* injected NOD/SCID mice intravenously with purified virus, which resulted in latent infection of leukocyte populations relevant to KSHV pathogenesis and low levels of spontaneous lytic reactivation that resulted in virus production. They also found that engraftment of human hematopoietic tissue into NOD/SCID mice prior to KSHV infection allowed for a KSHV-specific immune response. Infection of the chimeric mice was attenuated following treatment with systemic antiviral therapy (119).

In another study, An *et al* injected long-term infected telomerase-immortalized human umbilical-vein endothelial cells (TIVE) into nude mice. The tumors had many features that define KS tumors, including expression of high levels of LANA and lymphatic endothelial specific antigens found in KS. Furthermore, host genes, like those encoding IL-6, vascular endothelial growth factor, and basic fibroblast growth factor were also up-regulated (6).

Recent work done by Mutlu *et al* used the KSHV BAC (KSHVBac36) to transfect normal mouse bone marrow endothelial-lineage cells (mECs). This resulted in an angiogenic phenotype of the mECs and KS-like tumors that were dependent on maintenance of the KSHV genome (114). However, Bac36 transfection of mECs led to a nonproductive infection, further highlighting the limitations of KSHV models, in particular for studying viral entry and viral replication.

The development of these small animal models has certainly shed light on some aspects of KSHV biology, but mice are not small furry humans. Species differences in anatomy, metabolism, physiology or pharmacology are an issue, underlaid by further

species-specific genetic variations. To more closely examine the pathology of KSHV in humans, the development of non-human primate models will prove useful. At least one study has examined KSHV infection in rhesus monkeys, with or without co-infection of simian immunodeficiency virus (SIV) (133). It was found that a low-level of DNA replication occurred, but there was no disease progression, showing that the rhesus monkey is not a suitable animal model for KSHV.

Other Model Viruses

A number of closely related viruses to KSHV have been studied, with the hope of making parallel comparisons. These viruses naturally infect species other than humans. One such herpesvirus, herpesvirus saimiri (HVS), was isolated in the late 1960's from squirrel monkeys (107). This virus has served as a model for oncogenic herpesviruses. Although this virus does not cause disease in its natural host, it induces fatal acute T-cell lymphoma in other monkey species after experimental infection.

A mouse gammaherpesvirus has also been widely studied, murine gammaherpesvirus 68 (MHV-68). Isolated in 1990 (60), this virus originally served as a model for EBV. Like HVS, it undergoes lytic replication in tissue culture. Because MHV-68 naturally infects mice, it has served as an *in vivo* model of pathogenesis, allowing for the study of individual genes and their contribution to infection. But, MHV-68 is only partially related to KSHV at the genomic level, making for limited comparisons.

Macaque Rhadinoviruses

Investigators have identified a number of gamma-herpesviruses in macaque species. Rhesus Monkey Rhadinovirus (RRV) is by far the most studied and is detailed

below. In addition to RRV, two other rhadinoviruses have been identified. Initially found in tissue from macaques with simian retroperitoneal fibromatosis (RF), which is a rare vascular tumor that is similar to KS, these two new herpesviruses were designated as RF-associated herpesvirus (RFHV). The viruses, $RFHV_{Mn}$ and $RHFV_{Mm}$ were found in tissue from both *Macaca nemestrina* and *Macaca mulatta* species, respectively (139). Subsequent sequence analysis of these isolates revealed they are even more closely related to KSHV than RRV (139, 145). However, only about 7 kilobases from these viruses has been isolated. These viruses have never been found in live animals, and the limited availability of preserved RF tissue have prevented the isolation and further characterization of $RFHV_{Mn}$ and $RHFV_{Mm}$ (138).

Rhesus Monkey Rhadinovirus

The Discovery of Rhesus Monkey Rhadinovirus

In 1997, investigators at the New England Primate Research Center (NEPRC) noted that co-culturing of PBMCs from healthy rhesus monkeys with primary rhesus fibroblasts (RhF) led to cytopathic effect (CPE) in the fibroblasts. From these cells, the investigators were able to isolate a novel herpesvirus they designated rhesus monkey rhadinovirus (RRV), strain H26-95 (51). Almost simultaneously, investigators at the Oregon Regional Primate Research Center (ORPRC) isolated and sequenced a different strain of RRV, 17577, from SIV-infected macaques that had developed a lymphoproliferative disorder. They subsequently found that healthy animals in the same colony also harbored RRV-17577 (18). Sequence analysis of the two separate isolates demonstrated that the two strains were highly homologous, and at the time, were the most

closely related herpesvirus to KSHV. RRV and KSHV share nearly all of the same genes, with almost identical genomic organization and co-linear genomes (Figure 4).

In vivo and *in vitro* RRV infection

In captive rhesus macaques, RRV infection appears to be quite high, as many as 90% of animals are seropositive (18, 147). Infection rates among wild monkeys have not been documented. Experimental infection of naïve monkeys results in a transient lymphadenopathy and febrile illness lasting approximately 12 weeks. Antibody responses to RRV remain consistently high and virus can be isolated from peripheral blood. RRV DNA can be detected in lymph nodes, oral mucosa, skin and PBMCs and appears to preferentially infect CD20 positive B-cells (103, 165). It has also been found that some animals develop arteriopathy that resembles vascular endothelial lesions observed in KS patients. When co-infected with SIV, rhesus monkeys display an attenuated antibody response and a shorter mean survival time when compared to animals infected with SIV alone (103). In one study, co-infection with RRV and SIV led to a lymphoproliferative disorder similar to MCD and B-cell hyperplasia (165).

In vitro, RRV can be propagated by lytic infection of rhesus fibroblasts (RhF). This is a 100% lytic system, and high titer virus can be produced (53). This is in the context of *de novo* infection, allowing for analysis of viral replication using traditional plaque assay. Viral titers can also be determined using real-time quantitative PCR to measure viral genome copies (53). Latent/persistent systems of infection for RRV have also been developed, using transformed and immortalized B-cells (20, 52). Using either

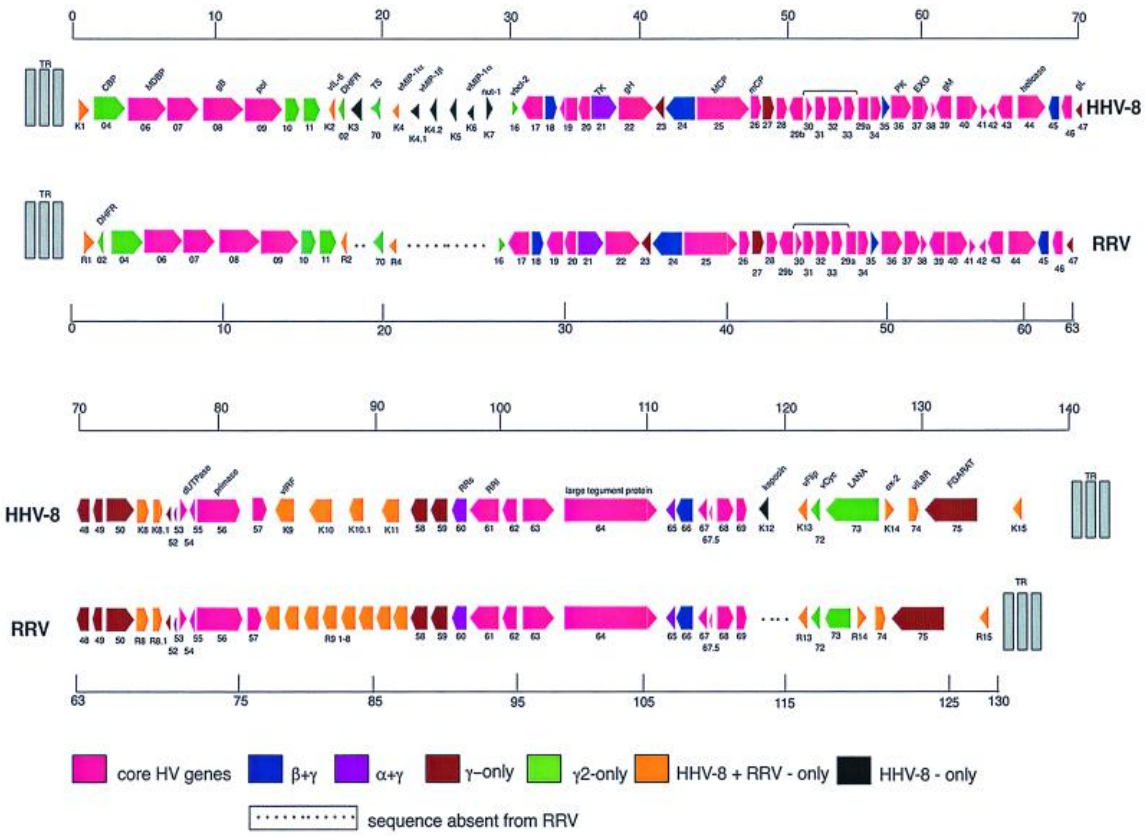

Figure 4. Alignment of RRV and KSHV genomes. ORFs are colored according the code at the bottom of the figure. Arrowheads indicate the 5' to 3' direction of coding region. Numbers designate each ORF and those preceded by K and R indicates uniqueness to KSHV and RRV, respectively. Lines connecting two ORFs designate spliced genes, and TR indicates terminal repeats. ORF size is approximated. (From Alexander *et al*(2))

TPA or RRV-ORF50, these cells can be induced to undergo lytic replication (52). Transcriptional mapping of RRV genes expressed during *de novo* lytic replication has been performed and the transcriptional program of RRV was found to closely resemble that of KSHV (57), further validating RRV as a model system for KSHV.

Genetic Manipulation of RRV

The ability of RRV to replicate in rhesus fibroblasts allows for the study of the contribution of individual open reading frames to overall viral replication. In order to do this, genetic manipulation of the viral genome is vital. The conventional means to do this is through homologous recombination in eukaryotic cells, which allows for the introduction of specific mutations in the viral genome. This method has been used to create a GFP-expressing RRV recombinant virus (53). In addition, there have been two recently described systems that allows for genetic manipulation of RRV: (i) creation of a bacterial-artificial chromosome (BAC) containing the RRV 17577 genome (61) and (ii) a set of overlapping cosmids incorporating the H26-95 genome (21). Using BAC-derived RRV, Estep *et al* infected SIV-positive rhesus macaques, which resulted in the induction of B cell hyperplasia, persistent lymphadenopathy, and persistent infection of RRV (61). By way of the cosmid system, Bilello and colleagues generated GFP- and secreted-engineered alkaline phosphatase (SEAP-) expressing RRV. With these viruses, they developed several assays to monitor RRV infection, neutralization, and replication. Sera from RRV-positive monkeys, but not RRV-negative monkeys, were consistently able to neutralize RRV infectivity when assayed by the production of SEAP activity or by the ability to express GFP (21). With the advent of these systems, genetic manipulation and characterization of individual ORFs of RRV will be greatly enhanced. Further

investigations into the mechanisms of replication and pathogenesis of RRV will help with our understanding of KSHV biology.

The R1 Open Reading Frame of RRV

Since the initial discovery of RRV, many of the KSHV homologous ORFs have been analyzed outside the context of the virus. This includes the K1 homologue, R1. Like K1, R1 is also encoded by the first ORF. The initial characterization of R1 showed sequence motifs and an organization of structural features similar to K1. R1 and K1 have approximately 27% amino acid identity in their extracellular domains, resembling those of the Ig receptor superfamily. R1 and K1 differ significantly in the length of their cytoplasmic domains. Unlike K1, which encodes two SH2-domain binding sites, R1 encodes for thirteen such sites, five of which fit the spacing pattern for an ITAM. R1 was shown to transform rodent fibroblasts and these cells induce multi-focal tumors in nude mice. R1 was also able to substitute for the STP oncogene of HVS to immortalize T lymphocytes to interleukin-2-independent growth (48).

Further characterization of R1 demonstrated that the cytoplasmic domain of R1 is capable of transducing signals that elicit B-lymphocyte activation events. This included induction of calcium release, induction of NFAT transcription factor, and phosphorylation of cellular tyrosine residues. R1 was also shown to interact with the major B-cell kinase, Syk, causing the phosphorylation of R1 itself (46).

OBJECTIVES

Expression of the K1 protein has been found in all KSHV-associated malignancies, albeit to different levels. This suggests that K1 plays an important role in KSHV pathogenesis. Although K1 itself has not been shown to transform primary B- or

endothelial cells *in vitro*, it has been shown to immortalize primary human endothelial cells (161) and deregulate normal signaling pathways in B and endothelial cells. The goal of this study was to characterize K1 signal transduction and expression and to functionally characterize R1, the RRV homologue of K1.

1) **Analyze K1 signal transduction in B-lymphocytes.**

   This study aims to determine K1's ability to activate the PI3K signal transduction pathway in B-lymphocytes. We also analyzed the role of K1 in Fas-mediated apoptosis.

2) **Determine if signaling by K1 is regulated by endocytosis.**

   There is currently no information about the regulation of surface expression of K1. The aim of this project was to determine whether K1 is internalized from the surface of cells and whether the signaling function of K1 can influence its internalization. We also looked at K1 internalization in B-lymphocytes and how this relates to BCR surface expression.

3) **Functional analysis of R1 in the RRV life-cycle.**

   The RRV homologue of K1 was identified in 1999 (48). This ORF, termed R1, has been shown to be functionally analogous to K1 as determined by signal transduction and activation of cellular transcription factors. In this study, we have deleted R1 from the RRV genome and analyzed the properties of the RRVΔR1 recombinant virus in the context of both lytic infection and the establishment of latency.

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CHAPTER TWO

THE K1 PROTEIN OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV) ACTIVATES THE AKT SIGNALING PATHWAY.

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV) has been implicated in Kaposi's sarcoma, as well as primary effusion lymphoma and multicentric Castleman's disease. The K1 protein of KSHV has been shown to induce cellular transformation, foci formation, and to deregulate B-lymphocyte signaling pathways by functionally mimicking the activated B-cell receptor complex. Here we show that expression of K1 in B-lymphocytes targets the phosphatidylinositol-3 kinase pathway leading to the activation of the Akt kinase and inhibition of the phosphatase, PTEN. We also demonstrate that activation of Akt by the K1 protein leads to the phosphorylation and inhibition of the forkhead (FKHR) transcription factor family, which are key regulators of cell cycle progression and apoptosis. We demonstrate that K1 can inhibit apoptosis induced by the FKHR protein and by stimulation of the Fas receptor. Our observations suggest that the K1 viral protein promotes cell survival pathways, and may contribute to KSHV pathogenesis by preventing virally infected cells from undergoing apoptosis prematurely.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) is a gammaherpesvirus that was first identified in Kaposi's sarcoma (KS) biopsies (13). KSHV has since been found in all epidemiological forms of KS. Viral DNA has been consistently isolated in AIDS-associated KS as well as HIV-negative classic and transplant-associated KS (20). KSHV has also been associated with lymphoproliferative diseases, such as pleural effusion lymphomas (PELs) and multicentric Castleman's disease (MCD) (51), both of which are B-cell in origin. However, the molecular mechanism by which KSHV induces malignancy in its host remains to be resolved.

At the far left-end of its genome, KSHV encodes a 46 kD transmembrane glycoprotein called K1. This position is equivalent to that of the herpesvirus saimiri (HVS) transforming protein (STP) and the R1 gene of rhesus monkey rhadinovirus (RRV) (15). K1 expression has previously been shown to deregulate cell growth, inducing foci-formation and morphologic changes in Rat-1 fibroblasts (34). Additionally K1 can functionally substitute for STP of HVS in transforming common marmoset T lymphocytes to IL-2 independent growth (34). Furthermore, transgenic K1 mice develop tumors with features of spindle-cell sarcomatoid tumors and malignant plasmablastic lymphomas (42).

K1 is structurally similar to the B cell receptor (BCR). The cytoplasmic tail contains an immunoreceptor tyrosine-based activation motif (ITAM), which has been shown to be capable of activating signaling pathways (30, 33) similar to those activated by the BCR complex in B-lymphocytes. However, unlike BCR, it is thought that K1 is constitutively active through oligomerization via conserved, extracellular cysteine

residues (30). Activation of K1 leads to phosphorylation of the ITAM, and recruitment of the major B cell kinase, syk. This initiates a signaling cascade in K1-expressing B cells (30, 33). Additionally, the cytoplasmic tail of the K1 protein has been shown to induce the phosphorylation of several different signaling molecules including syk, vav, cbl, and the p85 subunit of phosphatidyl-inositol-3'-OH-kinase (PI3K) leading to lymphocyte activation as measured by calcium mobilization, phosphorylation of tyrosine residues, and up-regulation of the NFAT and AP-1 transcription factors (33). K1 has also been shown to inhibit intracellular transport of BCR complexes, and to have effects on viral lytic reactivation/replication (29, 31, 32). Furthermore, lymphocytes isolated from transgenic mice expressing K1 showed constitutive activation of NF-κB and Oct-2, as well as enhanced Lyn kinase activity (42).

The PI3K/Akt pathway is one of the key signaling pathways activated upon engagement of the BCR complex. PI3Ks are heterodimeric enzymes consisting of a regulatory subunit, p85 and a catalytic subunit, p110 (10). Consequent to BCR activation, p85 is recruited to the BCR-Syk complex, followed by the recruitment of the p110 subunit (4, 50). This results in activation of PI3K and phosphorylation of the lipid-membrane associated moiety phosphatidylinositol 4,5-bisphosphate (PIP2) to yield phosphatidylinositol 3,4,5-triphosphate (PIP3). The Akt kinase specifically binds PIP3 through its pleckstrin homology (PH) domain, and this event recruits Akt to the plasma membrane (2, 21, 55). The PTEN phosphatase has previously been shown to be a negative regulator of this pathway (52, 58). PTEN catalyzes dephosphorylation at the D3 position of PIP3, serving to counter the effects of PI3K, and resulting in an inhibition of Akt. Conversely, PTEN is itself inactivated by phosphorylation, leading to the activation

of Akt kinase (56, 57). Active Akt kinase promotes cellular survival mechanisms, by directly phosphorylating and inactivating pro-apoptotic factors such as BAD and caspase-9 (9, 16, 18). Additionally, Akt phosphorylates a family of transcription factors known as the forkhead transcription factors (FKHR)(7, 27, 54). Members of this family include FKHR, FKHRL1 and AFX. The net result of phosphorylation of the downstream targets of Akt is cell survival via inactivation of the FKHR family, GSK-3β, Caspase-9 and Bad (9, 14, 16, 18).

At the present time, the exact mechanism by which the KSHV K1 viral protein transforms cells and activates B cell signaling pathways remains to be elucidated. Given the fact that K1 elicits B cell signaling events and that the cytoplasmic tail of K1 can induce the phosphorylation of a number of different kinases, including the p85 sub-unit of PI3K (33), we attempted to dissect the downstream effects of K1 signaling. Here we show that K1 up-regulates the PI3K pathway in B-lymphocytes, resulting in the phosphorylation of Akt and PTEN. Further, this event appears to be significantly dependent on an intact K1 ITAM motif. Activation of Akt leads to an increase in phosphorylation of FKHR transcription factors. Phosphorylation of FKHR family members promotes their nuclear exclusion and inhibits their transcriptional activation properties (7, 27, 54). FKHR family members modulate transcription of several classes of genes involved in cell-cycle regulation, including $p27^{Kip}$ (36, 38), p130 (28), and cyclin-D1 (46), as well as genes that promote cell death, including Bim (19) and Fas ligand (7). We present data demonstrating that expression of the K1 viral protein in B-lymphocytes enhances cell survival signals and protects cells from FKHR- and Fas- mediated apoptosis.

MATERIAL & METHODS

Plasmid constructs: EF-K1 was constructed as previously described (30). The CD8 signal peptide and Flag M2 epitope was fused in frame to the amino-terminal end of the K1 open reading frame. Three K1 mutants, EF-$K1_{YY/FF}$, EF-$K1_{Y282F}$ and EF-$K1_{ITAM-}$ mutants were created using QuikChange Site-Directed Mutagenesis Kit from Stratagene. The tyrosines at positions 270, 271(EF-$K1_{YY/FF}$, EF-$K1_{ITAM-}$) and 282 (EF-$K1_{Y282F}$, EF-$K1_{ITAM-}$) from EF-K1 were changed to phenylalanine and verified by sequencing. pGL2-3xIRS, pcDNA3-FKHR, pcDNA3-GFP-FKHR and pcDNA3-GFP-$FKHR_{AAA}$ have been previously described elsewhere (38). pCDNA3-Bcl-2 was a gift from Dr. John Reed.

Cell lines, cell culture and transfections: BJAB cells, a human B-cell line that is KSHV- and EBV-negative, were maintained in RPMI 1640 medium supplemented with 10% Calf Serum, penicillin and streptomycin. 40 μg of EF-K1 plasmid or empty vector were electroporated in serum-free media into BJABs at 300V and 950μF. 24 hours post-electroporation, cells were rinsed in PBS and transferred to serum-free media for an additional 48 hours. For luciferase assays, 293 cells were transfected with 1μg of pcDNA3-GFP-FKHR or pcDNA3-GFP-$FKHR_{AAA}$ plus 2μg of pCDEF3-K1 or empty vector using GenePorter 2 reagent (Gene Therapy Systems) as directed by the manufacturer. Cells were harvested 48 hours post electroporation and luciferase assays were performed as previously described (6). BJAB cells were electroporated with 5 or 10 μg EF-K1 or empty vector EF, and 5 μg pCDNA3-FKHR or pCDNA3. A similar procedure was performed for transfection of BCBL-1 cells with the GenePorter 2 reagent.

Cell fractionation: BJAB cells were electroporated and incubated as described above. 72 hours post-electroporation, cells were harvested and fractionated into cytoplasmic and

nuclear lysates using OptiPrep (Nycomed Pharma) as described by the manufacturer with the following modifications: harvested cells were rinsed in PBS, pelleted and resuspended in 350uL cold buffer A (10mM HEPES [pH 7.8], 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 1mM PMSF, Complete Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail [Sigma]) and incubated on ice for 15 min prior to the addition of 40μL 10% NP40. Samples were vortexed, centrifuged and cytoplasmic supernatants were reserved. Pelleted nuclei were rinsed in homogenization buffer (0.25M Sucrose, 25mM KCl, 5mM $MgCl_2$, 20mM Tris [pH 7.8], phosphatase and protease inhibitors) and were resuspended in a final volume of 400 μL in homogenization buffer. Nuclei were isolated by underlying with 600 μL 30% OptiPrep and 800 μL 35% OptiPrep and centrifuged @ 10,000 rpm for 20 minutes. Isolated nuclei were rinsed in homogenization buffer, pelleted and lysed in NE buffer (20mM Tris [pH 8.0] 420mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 25% Glycerol, 0.5mM PMSF, protease and phosphatase Inhibitors) Lysates were incubated on ice for10 min and centrifuged to pellet debris. Supernatants were reserved as nuclear lysates.

Immunoblotting and Immunofluorescence: 72 hours post-transfection, cells were harvested and lysed in RIPA buffer containing phosphatase inhibitor cocktail and protease inhibitors. For K1 expression, cells were freeze/thawed 3 times. Protein concentrations were determined by Bradford Assay. 80μg of protein were loaded per lane and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Each blot was stained with Ponceau S to ensure that equivalent amounts of protein samples were loaded. Primary antibodies were used at 1:1000 and secondary antibodies were used at 1:2000. K1 expression was verified using

HRP-conjugated anti-Flag M2 (Sigma). Anti-Akt, anti-phospho-Akt (T308), anti-phospho-Akt (S473), anti-PTEN, anti-phospho-PTEN, anti-FKHR, anti-phospho-FKHR (S256), anti- phospho-PDK1, anti-phospho-GSK3β, anti-phospho-Bad and HRP conjugated anti-mouse and anti-rabbit antibodies were all purchased from Cell Signaling technologies. Anti- PI3K (p85) antibody and the anti-phosphotyrosine-HRP conjugated antibody (4G10) were obtained from Upstate Biotechnology. Anti- Caspase 9 antibody was a gift from M. Deshmukh. Immunoblotting detection was performed using the ECL plus kit (Amersham).

For Immunofluorescence assays, 293 cells were transfected as above, rinsed with TBS and fixed at room temp in methanol:acetone (1:1), rinsed with TBS and incubated for one hour with anti-FLAG M2-Cy3 (Sigma) at room temperature. Cells were rinsed with TBS and viewed using fluorescence Leica Axiovert microscope.

Apoptosis Assay: FKHR mediated apoptosis: 293 cells were transfected with the indicated amounts of EF-K1 or EF vector, 3 μg of pCDNA3-FKHR and 1 μg β-galactosidase expression vector using Superfect (Qiagen). Cells were harvested and analyzed 72 hours post-transfection. Fas-mediated apoptosis: 293, BJAB and BCBL-1 cells were transfected with the indicated amounts of EF-K1 and EF. Superfect (Qiagen) was used for transfection of 293 cells, electroporation was used for transfection of BJAB cells and Geneporter 2 reagent was used for transfection of BCBL-1 cells. 48 hours post transfection 1.0 mg/ml of anti-Fas antibody (Upstate Biotechnology) was added in 1% FBS and cells were harvested 24 hours later. Apoptosis was analyzed using ApoAlert Caspase-3 Colorimetric Assay Kit from Clontech. Transfection efficiency was normalized to β-gal expression (Galacto-Star, Tropix).

TUNEL Assay. ***Flow Cytometry*:** BJAB cells were electroporated with 20 micrograms of EF or EF-K1 expression plasmid. 48 hours post-electroporation, anti-Fas was added at 1μg/ml in RPMI with 1% FBS and incubated for 24 hours. Cells were then stained for TUNEL using the *In Situ* Cell Death Detection Kit (Roche) as directed by the manufacturer. Briefly, cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% TritonX-100 in 0.1% sodium citrate and incubated with the TUNEL reaction mixture for one hour. Cells were rinsed and analyzed by FACScan (Becton-Dickinson) and data acquired using Cytomation, Inc.

RESULTS

K1 activates the Akt signaling pathway. The KSHV K1 protein has previously been shown to induce the phosphorylation of the p85 subunit of PI3K (33). BJAB cells were transfected with EF or EF-K1 expression plasmids. Cells were harvested and cell lysates were subjected to immunoprecipation using an anti-p85 antibody to pull down PI3K. Immunoprecipitates were run on SDS-PAGE and a western blot was performed using an anti-pTyr-HRP antibody. As shown in Fig 1A, we observed an increase in the phosphorylation of the p85 sub-unit of PI3K in K1-expressing cells as previously described (33).

To understand the functional consequence of this induction, we analyzed the effects of K1 expression on downstream effectors in the PI3K pathway. We transfected BJAB B cells with either an EF-K1 expression plasmid or the empty vector, EF. Equal amounts of total protein per sample were subjected to SDS-PAGE and Western blot analyses were performed. Each blot was also stained with Ponceau S to ensure that equivalent amounts of protein samples were loaded and transferred. As shown in Fig.1B,

EF and EF-K1 transfected cells have equal amounts of the p85 subunit of PI3K and total amounts of PTEN. However, only the K1-expressing cells show a significant increase in amounts of phospho-PTEN on Serine 380, as measured by the phospho-specific anti-PTEN antibody. Phosphorylation of PTEN at Serine 380 is known to inhibit it from actively dephosphorylating PIP3. It has been shown that this inhibition allows for an increase in PIP3, which recruits Akt to the membrane, where it can be activated by the phosphoinositide-dependent kinase 1 (PDK1) kinase (2, 21). Interestingly, although we observed no increase in the phosphorylation of PDK1 itself, we did see an increase in phosphorylation of Akt on residues Serine 473 (S473) and Threonine 308 (T208) in K1-expressing cells (Fig. 1B). It has been widely reported that phosphorylation of Akt correlates with its activation (21, 26). Hence, expression of K1 in B cells resulted in the activation of Akt while the total levels of this kinase remained unchanged (Fig. 1B). Upon dual phosphorylation, Akt has been previously demonstrated to become fully activated, detach from the plasma membrane and phosphorylate target substrates such as Bad, forkhead transcription factors (FKHR), glycogen synthase kinase-3 β (GSK-3β) and Caspase-9 (7, 9, 14, 16, 18, 27, 38).

Expression of K1 results in the phosphorylation of the FKHR transcription factor family. Since K1 activated the Akt kinase in B cells, we investigated effects on the downstream targets of Akt. As shown in Fig. 2, of the four Akt targets we analyzed, only the phosphorylation of FKHR is increased in K1-expressing cells. We have seen similar effects of K1 expression on FKHR in 293 cells (data not shown). Finally, there was a very marginal increase in the phosphorylation of the pro-apoptotic protein, Bad (Fig. 2). To determine if the changes in protein expression and phosphorylation states in K1-

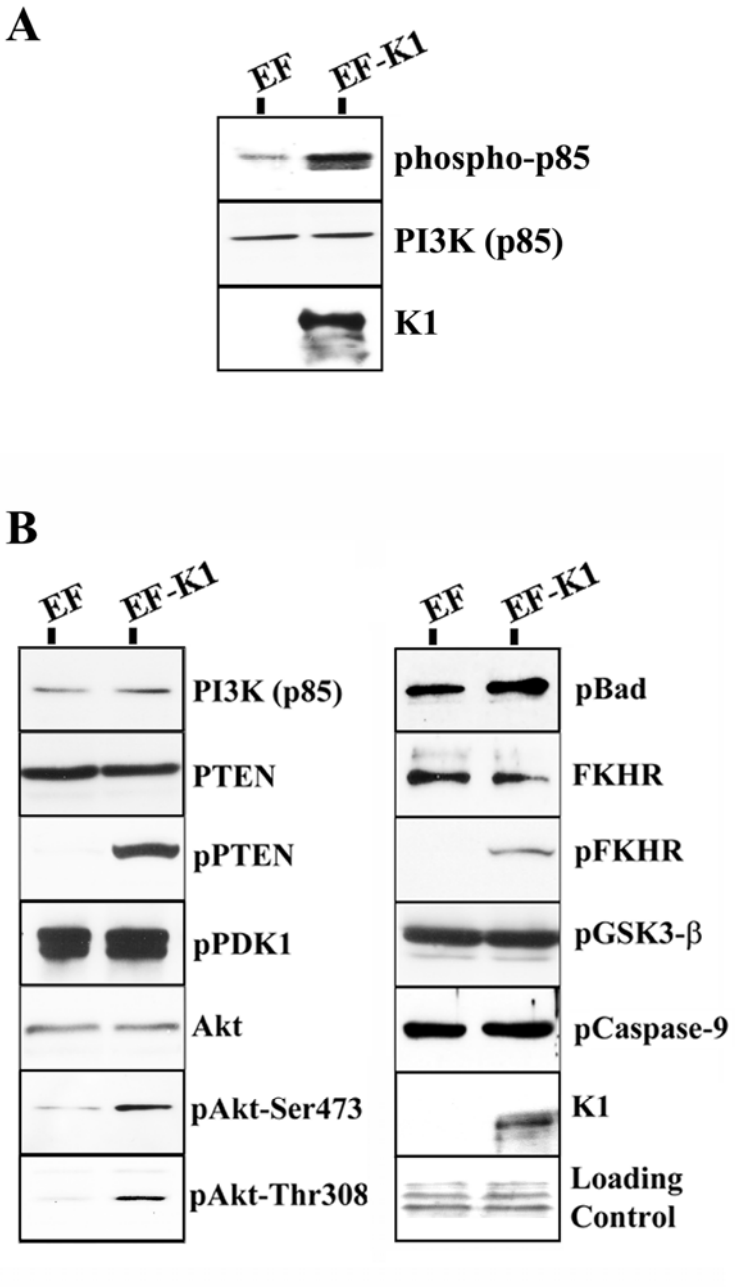

Figure 1. K1 activates the Akt pathway in B cells. A. BJAB cells were transfected with EF or EF-K1 expression plasmids as indicated. Cells were harvested and lysed and subjected to immunoprecipitation with an anti-p85 antibody to pull down PI3K. A western blot analysis was performed on the immunoprecipitate reactions using an anti phospho-Tyr-HRP antibody to detect the phosphorylated p85 sub-unit of PI3K. **B**. BJAB cells were transfected with empty vector (EF) or a K1 expression vector (EF-K1). Equal amounts of proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies. Ponceau S staining was used to evaluate equivalent loading of the samples. K1 expression was determined by probing with an anti-FLAG antibody.

expressing B cells are directly attributable to activation of Akt by PI3K, we inhibited PI3K activation by using the specific chemical inhibitor LY294002. BJAB cells were transfected as described above, and LY294002 was added to the culture for a period of 12 hours as previously reported (40). Upon exposure to LY294002, phosphorylation of Akt at Serine 473 is completely eliminated while phosphorylation of Akt at Thr 308 is inhibited slightly. It has been shown that the phosphorylation of Akt at S473 is much more sensitive to PI3K inhibition by LY294002 than the T308 residue (35) which may account for the different phosphorylation states of the two residues in this experiment. Phosphorylation of Akt re-appears after an additional 12 hours (Fig.2, 24 hr panel), and this is most likely due to the drug losing its effect. There is no inhibition of PTEN phosphorylation. We also see a slight inhibition of FKHR phosphorylation after twelve hours of exposure to LY294002 in K1-expressing cells and similar to Akt, this is reversed at 24 hours. The total levels of FKHR remain unaffected by the presence of LY294002. Curiously, LY294002 does not affect the phosphorylation of Bad. However, it has been shown that there are other kinases in addition to Akt that are capable of phosphorylating Bad (61).

Activation of the PI3K/Akt pathway and phosphorylation of FKHR by a panel of K1 mutants. A lot of importance has been given to the presence of the immunoreceptor tyrosine based activation motif or ITAM, present in the cytoplasmic tail of K1. Despite significant variation in K1 sequences from different KSHV isolates around the world, more than 60 K1 isolates sequenced to date show that the ITAM motif in the K1 cytoplasmic domain is always conserved (64). It has been demonstrated that the K1 ITAM is capable of activating signaling pathways (30, 33) similar to those activated by the BCR complex in B-lymphocytes. In order to investigate whether the K1 ITAM motif was responsible for activating the Akt signaling cascade described above, we analyzed three mutants of K1 in which the tyrosine residues in the ITAM were individually or dually mutated to phenylalanine. These mutants were cloned into the same EF vector as

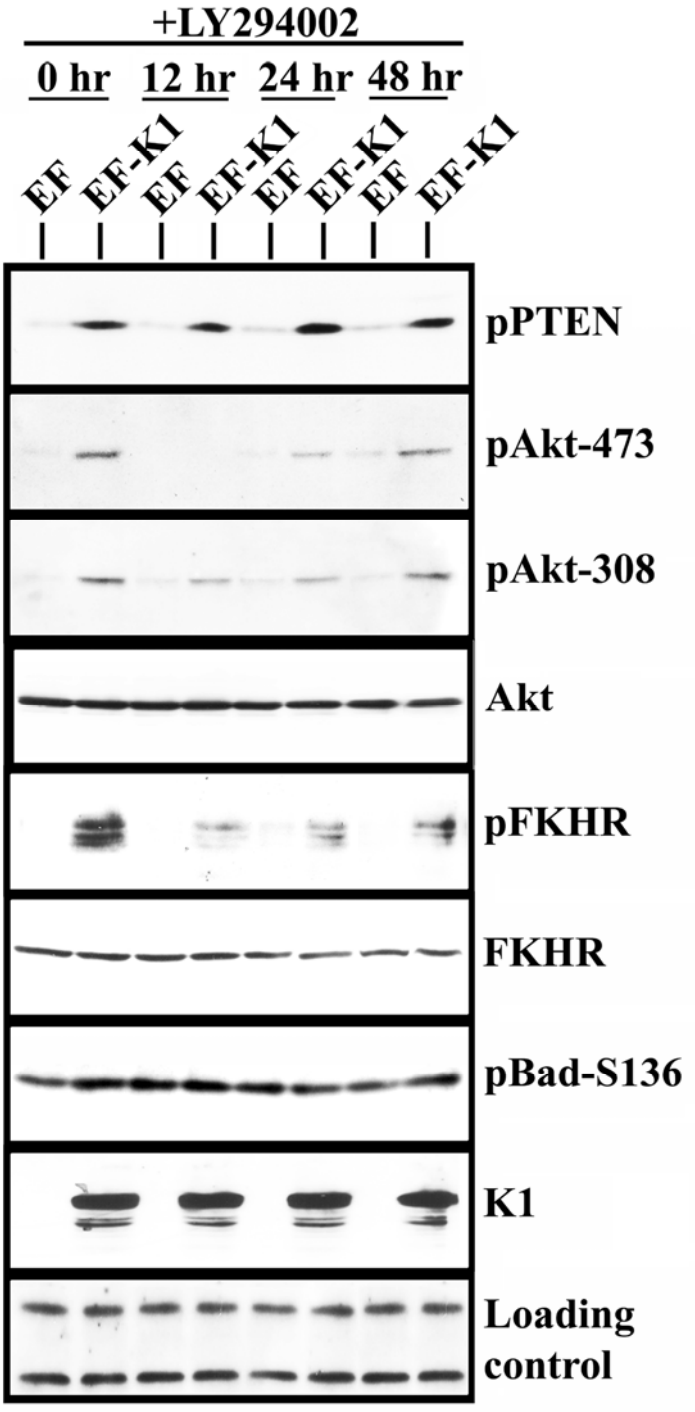

Figure 2. Activation of Akt by K1 is inhibited by LY294002. EF or EF-K1 transfected BJAB cells were incubated for 12 or 24 hours in the presence of 10μM LY294002. Lysates were subjected to Western blot analysis and probed with the indicated antibodies.

wild-type K1, and were named EF-K1$_{Y282F}$, EF-K1 $_{YY/FF}$ and EF-K1 $_{ITAM}$- respectively. We next determined whether these tyrosine-altering mutations in the K1 cytoplasmic tail were capable of activating the Akt pathway in BJAB B cells. Cells were electroporated with the aforementioned expression plasmids and western blots were performed to analyze the phosphorylation status of PTEN, Akt and FKHR. As shown in Fig. 3, all three K1 mutants, K1$_{Y282F}$, K1 $_{YY/FF}$ and K1 $_{ITAM}$- , were unable to phosphorylate PTEN and thus unable to inactivate PTEN as compared to wild-type K1. In addition, analysis of the phosphorylation and activation state of Akt suggested that wild-type K1 and to some extent, the K1 $_{YY/FF}$ mutant could still activate Akt as measured by phosphorylation of T308, but the K1$_{Y282F}$ and K1 $_{ITAM}$- mutants could not activate this kinase. Lee et al. had previously shown that the K1$_{YY/FF}$ mutant could weakly induce cellular tyrosine phosphorylation, although it was at a much reduced level as compared to wild-type K1 (33). The same pattern was seen when we looked at the FKHR transcription factor (Fig. 3). Wild-type K1 and to some degree, the K1 $_{YY/FF}$ mutant could still induce inactivation of FKHR as measured by phosphorylation of FKHR, but the K1$_{Y282F}$ and K1 $_{ITAM}$- mutants could not inactivate FKHR (Fig. 3).

Expression of K1 results in the cytoplasmic sequestration of FKHR. Direct phosphorylation of FKHR by Akt has been previously shown to result in cytoplasmic retention and inactivation of forkhead transcription family members (5, 38, 54). We attempted to determine the cytoplasmic versus nuclear localization of FKHR in response to K1 expression. As we were limited by the rounded morphology of B cells, we analyzed localization of FKHR in K1-expressing 293 cells, which have a flatter morphology and a more demarcated nuclear and cytoplasmic compartment as compared

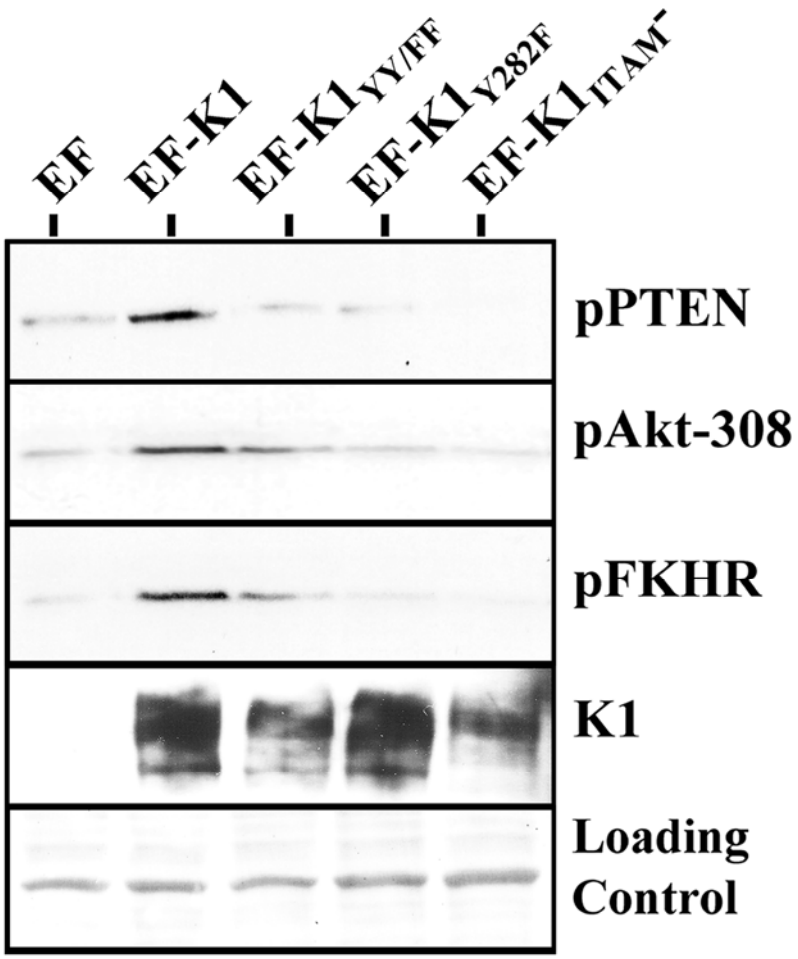

Figure 3. Activation of the PI3K/Akt pathway by K1 mutants. BJAB cells were electroporated with the indicated expression plasmids. Cells were harvested as previously described. Equal amounts of proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies. The individual panels from top to bottom represent western blot performed using an anti-pPTEN, anti-pAKT (T308) and anti-pFKHR antibody, respectively. Ponceau S staining was used to evaluate equivalent loading of the samples. K1 expression was determined by probing with an anti-FLAG antibody.

to B cells. 293 cells were co-transfected with either empty vector EF, or EF-K1 plasmid, as well as a FKHR-Green Fluorescent Protein (GFP) fusion protein expression plasmid (38, 54.). In Fig. 4, panels a and b depict the localization of FKHR-GFP in the presence of empty vector, EF. The FKHR-GFP protein resides in both the cytoplasm and the nucleus, with the majority of the protein in the nucleus. This correlates with what has been previously shown for this protein (38). Since FKHR in the nucleus activates transcription of pro-apoptotic genes, these cells also appear to be undergoing apoptosis as is evident from their morphology. However, in cells where FKHR-GFP was co-expressed with K1 (Fig.4, panels c, d, e, f), the FKHR-GFP protein was predominantly excluded from the nucleus (see arrow in Fig. 4 panels d, e, f). This was true for the majority of cells expressing both FKHR-GFP and the K1 protein. This conclusion is strengthened by the observation that the adjacent cell, which does not express K1, has FKHR–GFP localized to both the nucleus and cytoplasm and appears to be undergoing apoptosis. To determine if the localization of FKHR is dependent on the phosphorylation of FKHR by Akt, a mutant FKHR ($FKHR_{AAA}$) lacking the three Akt phospho-acceptor sites Threonine24A, Serine256A, and Serine319A (38, 54) was co-transfected along with the EF-K1 expression vector (Fig. 4, panels g, h, i, j). The $FKHR_{AAA}$ –GFP protein was predominantly localized in the nucleus, regardless of whether or not the cell was also expressing K1 (Fig. 4, panels g, h, i, j), thus corroborating our observation that K1's effects on FKHR are dependent on Akt phosphorylation of the three aforementioned phospho-acceptor sites. Finally, we also analyzed the ability of the K1 mutant, $K1_{ITAM}$-, and found that it was unable to sequester the FKHR-GFP fusion protein in the cytoplasm of the cell (Fig. 4, panels, k,l,m,n). Thus suggesting that FKHR phosphorylation and

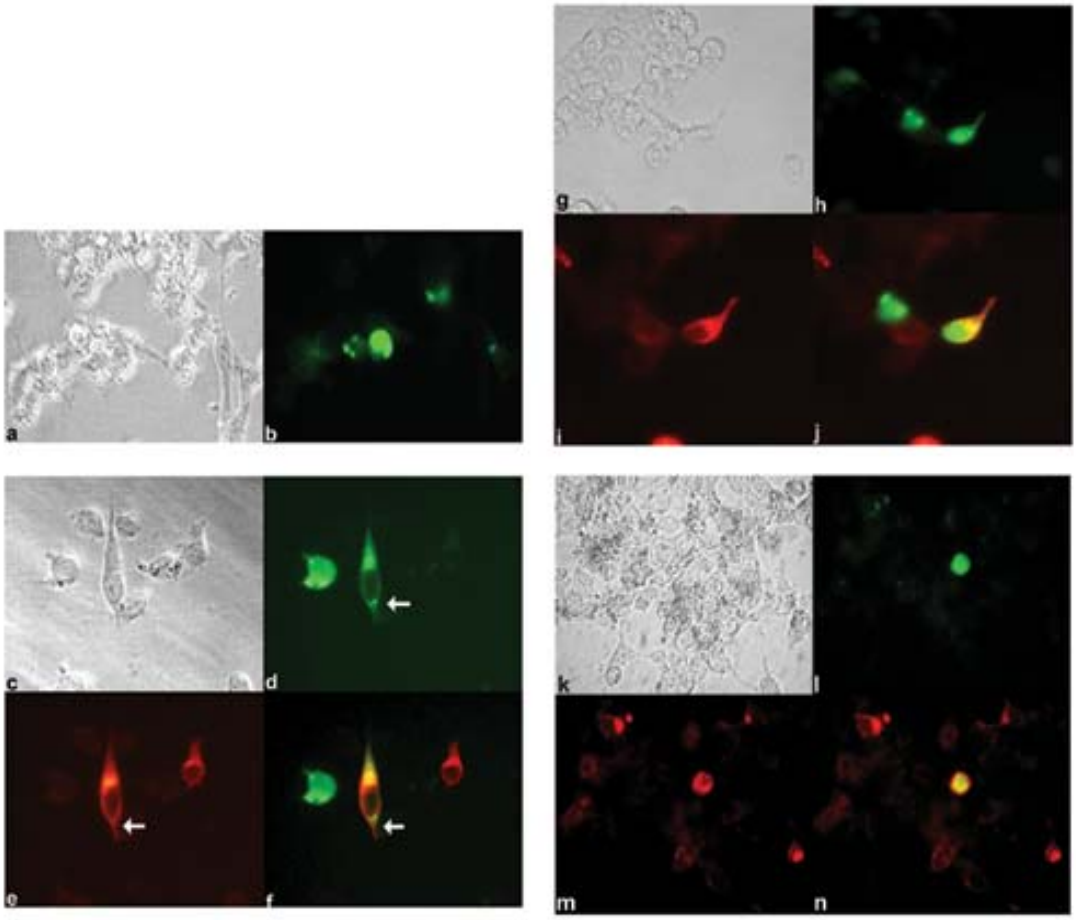

Figure 4. K1 promotes cytoplasmic localization of FKHR. **a**. 293 cells were transfected with empty-vector (EF) and FKHR-GFP. Transfected cells were fixed and examined under bright-field microscopy. **b.** Panel represents the same cells as in panel a, that were examined for expression and distribution of FKHR-GFP (green) under immunofluorescence microscopy in empty vector (EF) and FKHR-GFP co-transfected cells. Cells expressing FKHR-GFP appear to be undergoing apoptosis based on their rounded morphology. **c**. Cells were transfected with EF-K1 and FKHR-GFP expression plasmids. **d.** Panel represents the same cells as in panel c, which were fixed and examined for expression and distribution of FKHR-GFP (green) **e**. The same cells as depicted in panels c and d, were stained for expression of K1 (red) and are shown under immunofluorescence microscopy. f. A merged image of the same cells shown in panels **c, d**, and **e.** Yellow represents the co-localization of K1 (red) and FKHR-GFP (green). The white arrow indicates a cell that is co-expressing K1 and FKHR-GFP. The adjacent cell to its left expresses FKHR-GFP but is not expressing K1 and appears to be undergoing apoptosis. **g**. Cells were transfected with EF-K1 and the mutant $FKHR_{AAA}$-GFP expression plasmids. **h.** The same cells shown in panel **g**, were analyzed for expression and distribution of $FKHR_{AAA}$-GFP (green). Based on their morphology, these cells appear to be undergoing apoptosis i. Cells were examined for expression of K1 (shown in red) under immunofluorescence microscopy**. j**. A merged image of the same cells shown in panels **g, h**, and **i**. Yellow represents the co-localization of $FKHR_{AAA}$-

GFP (green) and K1 (red). **k**. Cells were transfected with FKHR-GFP and the EF-$K1_{ITAM}$- expression plasmids. Transfected cells were fixed and examined under bright-field microscopy. **l.** The same cells shown in panel k were analyzed for expression and distribution of FKHR-GFP (green). m. Cells were examined for expression of the $K1_{ITAM}$- mutant (shown in red) n. A merged image of the same cells shown in panels **k, l,** and **m**. Yellow represents the co-localization of FKHR-GFP (green) and the $K1_{ITAM}$-mutant (red).

localization in K1 expressing cells is dependent on the ITAM motif.

To confirm the localization of endogenous FKHR in B cells in response to K1 expression, we transfected BJAB cells with either EF vector or the EF-K1 expression plasmid. Seventy-two hours post-transfection, cells were harvested and lysed. Cell lysates were subjected to a nuclear and cytoplasmic fractionation scheme as described in the Methods section and Western blot analyses were performed (Fig. 5). The fractions were analyzed for both total FKHR and phosphorylated FKHR. The amount of phospho-FKHR present in the nucleus of K1-expressing cells was lower compared to the empty vector transfected cells (Fig. 5, left panel). This is further supported by the observation that K1 expression results in an increase in phospho-FKHR protein in the corresponding cytoplasmic fraction of K1-expressing cells as compared to empty vector controls (Fig. 5, middle panel). Taken together, our data suggest that the majority of FKHR in K1-expressing cells is phosphorylated on residue S256, resulting in the cytoplasmic retention of FKHR and thereby causing an inhibition of its transcriptional activity (7, 24, 27). GRP78, a cytoplasmic protein, was used as a marker to assess the purity of the nuclear and cytoplasmic fractions.

K1 represses FKHR-responsive promoters and can inhibit both FKHR- and Fas-mediated apoptosis. The localization and phosphorylation of FKHR suggests that the transcriptional function of FKHR may be inhibited in K1-expressing cells. The observation that FKHR was being retained in the cytoplasm by expression of K1 in B lymphocytes prompted us to investigate whether K1 could prevent the activation of the FKHR family-responsive promoters. FKHR has been shown to activate transcription from a minimal promoter element contained within the insulin like growth factor binding

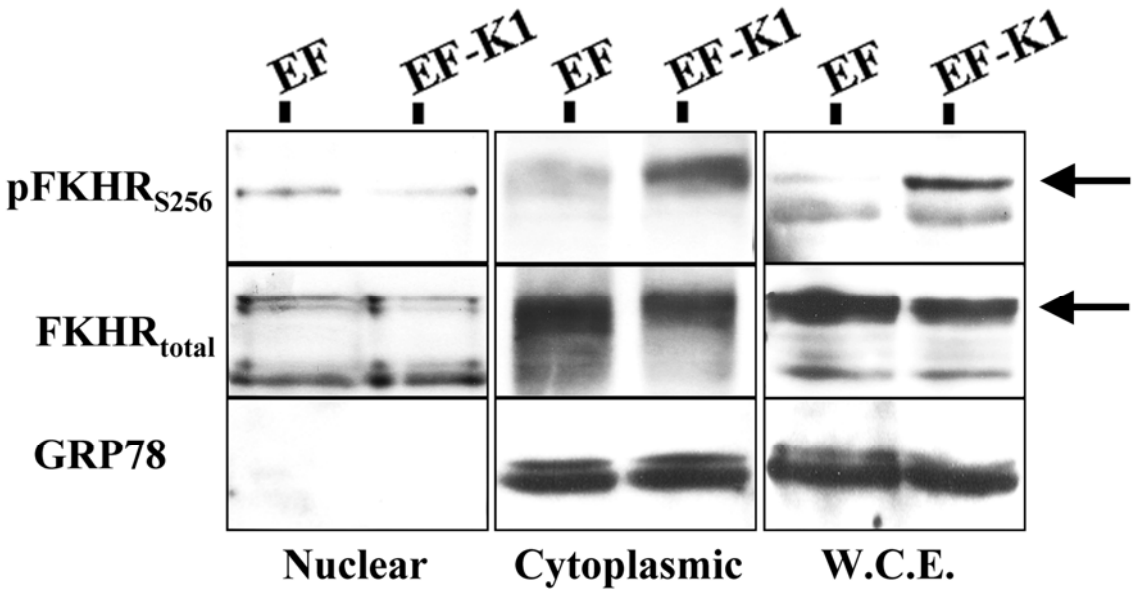

Figure 5. **Phospho-FKHR is retained in the cytoplasm of K1-expressing B cells.** BJAB cells were transfected with EF or EF-K1 and whole cell extract (W.C.E.) was fractionated into a nuclear and a cytoplasmic fraction. Equal amounts of cytoplasmic and nuclear fraction from each sample were subjected to Western blot analysis with the indicated antibodies. The left panel is the nuclear fraction. Middle panel is the cytoplasmic fraction and the right panel is whole cell extract. GRP78, a cytoplasmic protein, was used as a marker to assess the purity of the nuclear and cytoplasmic fractions. The arrows point to the FKHR-specific band.

protein-1 (IGFBP-1), known as the insulin response sequence (IRS), and a Forkhead-responsive element (FHRE) within the FasL promoter (7, 24, 54). Cells were co-transfected with varying amounts of EF-K1 or EF empty vector and a luciferase reporter plasmid FHRE-Luc that contains three Forkhead-responsive elements upstream of luciferase. A β-gal expression vector was used to control for transfection efficiency. K1 repressed the FHRE-Luciferase promoter three-fold (Fig. 6a). These three Forkhead-responsive elements are naturally present in the FasL promoter itself (7, 38) and are activated upon Fas receptor ligation. We next performed the identical experiment described in 6a, but stimulated the transfected cells with an anti-Fas receptor antibody to simulate the Fas receptor-dependent death pathway (Fig. 6b). K1 inhibited the FHRE-Luciferase promoter 4-fold in the presence of Fas-receptor engagement (Fig. 6b) suggesting that the K1 protein can protect against Fas-induced activation of pathways leading to cell death. Cells were also co-transfected with varying amounts of EF-K1 or EF empty vector and a different FKHR-responsive reporter plasmid, 3XIRS-luciferase, which is comprised of three insulin-response elements upstream of the luciferase reporter gene (7, 24, 27). Again, K1 repressed the 3XIRS-Luciferase promoter three-fold (Fig. 6c).

The Forkhead family of transcription factors has been implicated in cell survival through regulation of several pro-apoptotic genes, eg. FasL and Bim. Our observation that expression of K1 modulated FKHR expression and localization, as well as its transcriptional activity on the FHRE-Luciferase promoter lead us to investigate whether K1 could increase cell survival under conditions of apoptotic stimuli, such as over-expression of FKHR, as well as engagement of the Fas-receptor through FasL or anti-Fas

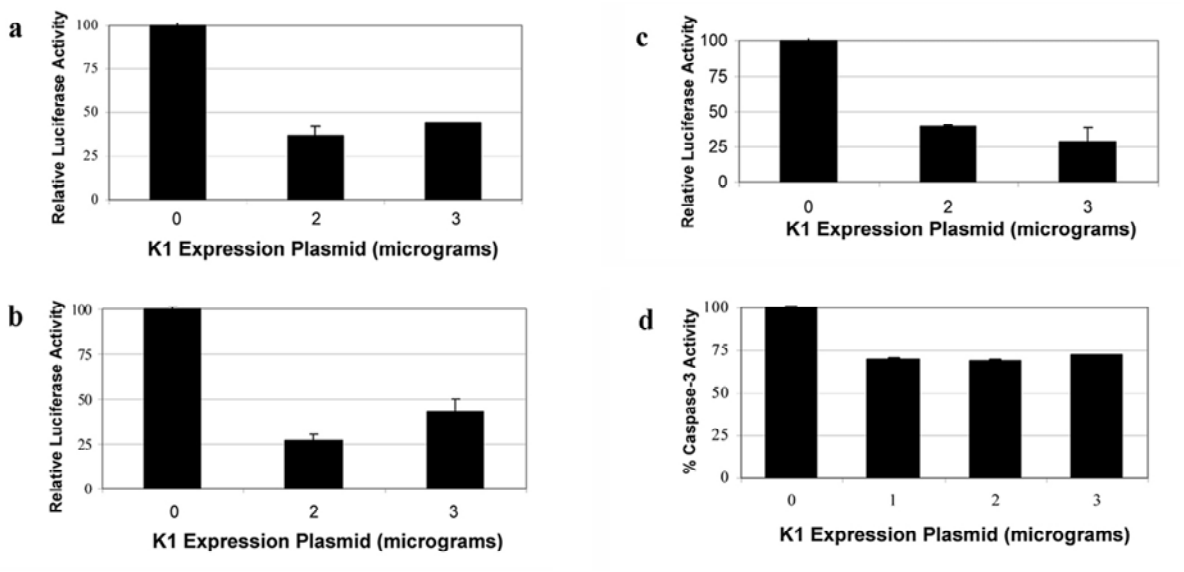

Figure 6. K1 represses forkhead-regulated promoters and protects cells from FKHR-mediated apoptosis. **a**. 293 cells were transfected with 0, 2 or 3 μg of EF-K1 or EF empty vector and 3ug of a 3XFHRE-luciferase plasmid. A β-gal construct was also co-transfected to normalize for transfection efficiency. 48 hrs post-transfection, cells were lysed and assayed for luciferase expression. Luciferase activity in the absence of K1 was set at 100% activity and relative luciferase activity in the presence of different amounts of the K1 expression plasmid was calculated as a percentage of this luciferase activity. Error bars represent the variation from the mean. **b.** Cells were transfected similar to panel a, except anti-Fas antibody was used to stimulate apoptosis and the FHRE-Luciferase promoter. Luciferase activity in the absence of K1 and presence of anti-Fas antibody was set at 100% activity and relative luciferase activity in the presence of different amounts of the K1 expression plasmid was calculated as a percentage of this luciferase activity. Error bars represent the variation from the mean. **c**. 293 cells were transfected with 0, 2 or 3 μg of EF-K1 or EF empty vector and 3ug of a 3XIRS-luciferase plasmid. Luciferase activity in the absence of K1 was set at 100% activity and relative luciferase activity in the presence of different amounts of the K1 expression plasmid was calculated as a percentage of this luciferase activity. Error bars represent the variation from the mean. **d**. Cells were transfected with 0, 1, 2, or 3 μg of EF-K1 or EF empty vector and pCDNA3-FKHR. 48 hrs post-transfection, cells were lysed and assayed for caspase-3 activity. Caspase-3 activity in the absence of K1 was set at 100% activity and relative caspase-3 activity in the presence of different amounts of the K1 expression plasmid was calculated as a percentage of this caspase-3 activity. Error bars represent the variation from the mean.

receptor antibody.

Caspase-3 is a downstream effector of both receptor-dependent and receptor-independent apoptotic stimuli and its activation has served as a marker for cells undergoing apoptosis as has been widely reported in the literature (41). We analyzed caspase-3 activity in EF versus EF-K1 expressing cells that were co-transfected with a pCDNA3-FKHR expression plasmid to induce FKHR-mediated apoptosis in these cells. We observed that expression of K1 in these cells resulted in a ~30% decrease in caspase-3 activity (Fig. 6d) thus indicating that K1 is able to thwart FKHR-mediated apoptosis. To determine whether K1 could inhibit Fas-mediated apoptosis, we transfected cells with EF, or EF-K1 vectors, pcDNA3 or pcDNA3-Bcl-2 vectors and 48 hours post-transfection stimulated these cells with anti-Fas receptor antibody. We observed that K1 expression resulted in a 50% decrease in caspase-3 activity induced by engagement of the Fas receptor as compared to cells transfected with EF vector alone (Fig. 7a). BJAB cells transfected with Bcl-2 showed an ~60% decrease in caspase-3 activity which corresponds well with published literature which demonstrated that Bcl-2 antagonizes Fas-receptior induced apoptosis in B lymphocytes (1). Finally, in order to determine whether K1 could protect from Fas-mediated apoptosis in KSHV-positive B cells, we repeated this experiment in BCBL-1 cells. We observed that expression of K1 in BCBL-1 cells resulted in a ~40% decrease in caspase-3 activity induced by engagement of the Fas receptor, while Bcl-2 resulted in a ~60% decrease in caspase-3 activity (Fig. 7b), again suggesting that K1 is able to inhibit Fas-death receptor dependent apoptosis. The $K1_{ITAM-}$ mutant was also tested in the caspase-3 cell death assays described above and only showed a 10% decrease in protection against Fas-mediated apoptosis (data not shown).

We also performed a TUNEL assay on BJAB cells transfected with either EF vector or EF-K1 expression plasmid (Fig. 7c). Cells were incubated with anti-Fas antibody for 24 hours to induce Fas-receptor dependent apoptosis and a terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL) assay was used to measure DNA fragmentation. BJAB cells were transfected with the EF or EF-K1 expression plasmid. 14.86% of cells transfected with EF vector alone were FITC or TUNEL-positive while only 7.5% of cells transfected with EF-K1 plasmid were FITC or TUNEL-positive by flow cytometry confirming our findings that K1 is able to protect against Fas-mediated apoptosis.

In order to confirm our findings, we also performed an *in situ* terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL) assay to measure DNA fragmentation. We found that 15% of cells expressing wild-type K1 stained positive for DNA fragmentation while 39% of cells expressing the $K1_{ITAM-}$ mutant protein stained positive by TUNEL. Thus, we conclude that wild-type K1 is able to protect cells from Fas-mediated apoptosis.

DISCUSSION

The KSHV K1 protein has been shown to have transforming potential by a wide variety transgenic animals and in nude mice injected with K1-expressing cells, and the ability of K1 to substitute for the STP oncogene in HVS to immortalize T cells to IL-2 independent growth and induce lymphomas in common marmosets (34, 42). Although K1 mRNA is induced upon lytic reactivation, its expression in latent cells cannot be definitively ruled out. K1 mRNA has been shown to be transcribed in KS lesions and in KSHV-infected B lymphoma cell lines, although in the latter case this may be a result of lytic K1

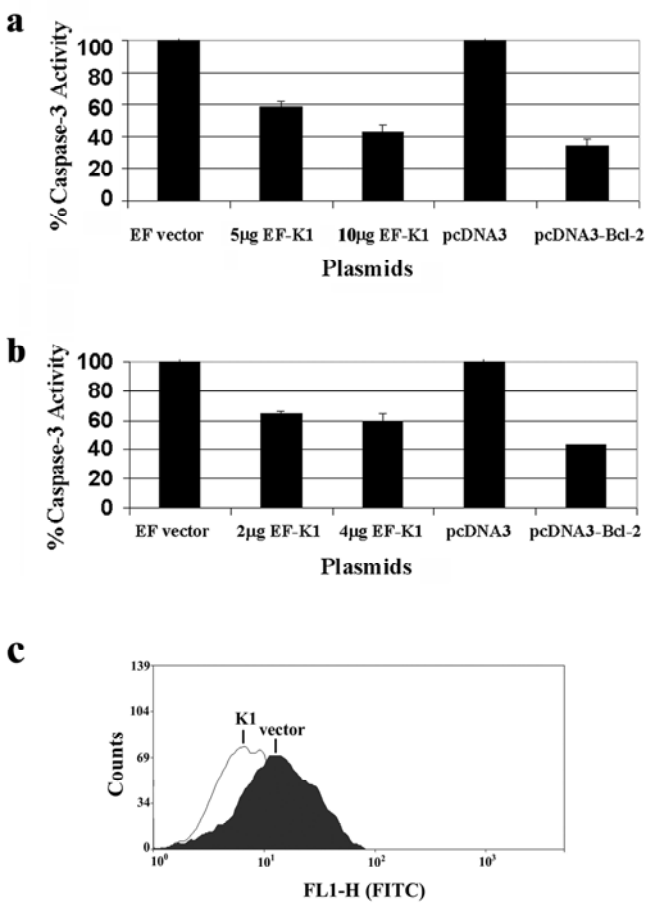

Figure 7. K1 protects cells from Fas-mediated apoptosis. a. KSHV-negative BJAB B cells were transfected with 0, 5 or 10 μg of EF-K1 or EF empty vector, or pcDNA3 or pcDNA3-Bcl-2 expression plasmid by electroporation. 48 hrs post-transfection, cells were stimulated with 1μg/ml anti-Fas antibody for 24 hours. Cells were lysed and assayed for caspase-3 activity. Caspase-3 activity in the absence of K1 was set at 100% activity and relative caspase-3 activity in the presence of different amounts of the K1 expression plasmid was calculated as a percentage of this caspase-3 activity. Error bars represent the variation from the mean. **b.** KSHV-positive BCBL-1 cells were transfected with 0, 2 or 4 μg of EF-K1 or EF vector, or pcDNA3 or pcDNA3-Bcl-2 expression plasmid using GenePorter 2 reagent. 48 hrs post-transfection, cells were stimulated with 1μg/ml anti-Fas antibody for 24 hours. Cells were lysed and assayed for caspase-3 activity. Caspase-3 activity in the absence of K1 was set at 100% activity and relative caspase-3 activity in the presence of different amounts of the K1 expression plasmid was calculated as a percentage of this caspase-3 activity. Error bars represent the variation from the mean. **c.** BJAB cells were transfected with the EF or EF-K1 expression plasmid. Cells were treated with anti-Fas antibody to simulate Fas-receptor dependent apoptosis. 24 hours later a TUNEL assay was performed. Cells were stained for fragmented DNA by enzymatically labeling the nicked ends with FITC-conjugated-dUTP and assayed by flow cytometry. 14.86% of cells transfected with EF vector alone were FITC or TUNEL-positive while only 7.5% of cell transfected with EF-K1 plasmid were FITC or TUNEL-positive.

expression in the 3-5% of cells that undergo spontaneous reactivation (6, 45). Regardless of whether K1 is expressed during latency, the expression of K1 during the KSHV lytic cycle may contribute to the paracrine stimulation that is thought to sustain proliferation in most KSHV-associated malignancies (12, 23, 45). It is possible that K1, similar to the KSHV vGPCR protein (12), activates expression of a number of cytokines and growth factors needed for expansion of KSHV-associated neoplasms. Hence, understanding K1's signaling properties is important to the understanding of KSHV pathogenesis.

The KSHV K1 protein bears a marked structural resemblance to members of the immunoreceptor super-family. Functional analysis of the K1 protein in B cells has demonstrated that K1 can elicit B lymphocyte signaling events. For K1 mediated activation, there is a requirement for the major B cell kinase, syk. In addition, K1 has been shown to induce the phosphorylation of several signaling molecules including the p85 sub-unit of PI3K. Given the fact that the PI3K pathway is linked not only to cell activation events but also to cell survival, our analysis here indicates that K1 is able to modulate the PI3K pathway by eliciting cell activation and survival responses.

In B lymphocytes, the Akt kinase is a downstream target of activated PI3K. Activation of Akt itself plays an important role in B-cell survival, proliferation and differentiation (40). Here we demonstrate that K1 expression leads to activation of Akt. When expressed in B-lymphocytes, K1 activates Akt by inducing phosphorylation at two separate residues, Thr308 and Ser473. Interestingly, K1 simultaneously appears to target the phosphatase, PTEN, which is a negative regulator of the Akt pathway. Expression of K1 results in increased PTEN phosphorylation, which is indicative of its inactivation.

Thus, K1 appears to activate Akt by a two-pronged approach that involves phosphorylation of the kinase itself as well as its regulatory phosphatase, PTEN. In addition, Akt activation by K1 was inhibited by the PI3K-specific inhibitor, LY294002, confirming that K1 modulates Akt via the PI3K pathway.

As a key regulator of cell survival events, Akt targets a number of different cytoplasmic proteins including GSK3β, Caspase-9, Bad and the FKHR family of transcription factors. As is widely reported, phosphorylation of these proteins by Akt generally results in their inactivation and inability to activate pro-apoptotic pathways. While K1 expression in B lymphocytes did not change phosphorylation of GSK3β, or Caspase-9, it resulted in the marked phosphorylation of FKHR transcription factors and altered their cellular localization. Curiously, the marginal inhibition of FKHR phosphorylation levels by LY294002 suggests that although K1 targets Akt, its affect on FKHR may be mediated by other kinases besides Akt. Indeed, it is known that serum-and glucocorticoid-inducible kinase (SGK) can also phosphorylate FKHR family members (8). A panel of K1 mutants in which the tyrosine residues in the ITAM were individually or dually mutated to phenylalanine ($K1_{Y282F}$, $K1_{YY/FF}$ and $K1_{ITAM}$-) were unable or very much reduced in their ability to activate Akt or inactivate PTEN and FKHR, suggesting that activation of this pathway is dependent upon the ITAM present in the K1 cytoplasmic tail to a significant extent.

A wide variety of reports have shown that the Akt kinase controls sub-cellular localization of the members of the FKHR family via phosphorylation. Phosphorylation of FKHR results in the preferential sequestration of these proteins in the cytoplasm, thus preventing them from activating their target genes such as FasL and Bim. The exact

mechanism for how this occurs has yet to be investigated. Phosphorylation may cause FKHR to interact with 14-3-3 proteins, resulting in nuclear export and cytoplasmic sequestration. It has also been observed that a decrease in DNA binding results when FKHR is phosphorylated on Ser 256 which is located in the FKHR DNA-binding domain (62). Similar to other laboratories, we observed that upon transient transfection of a FKHR-GFP expression plasmid into cells, FKHR localized mainly to the nucleus. However upon K1 co-expression in these cells, the FKHR protein was re-directed to the cytoplasm. This localization was dependent on phosphorylation since a $FKHR_{AAA}$-GFP mutant in which all three Akt phospho-acceptor sites were mutated to alanine accumulated in the nucleus, regardless of K1 co-expression. This observation was further supported by the fact that B-lymphocytes expressing K1 showed a decrease in the levels of phospho-FKHR in the nuclear fraction accompanied by a concomitant increase in phospho-FKHR in the cytoplasmic fraction. Interestingly, although we saw a specific effect of K1 expression on the FKHR family, we did not see an affect on other downstream targets of Akt such as GSK-3β and Caspase-9. This suggests that these targets may be regulated by counteractive signals which prevent their phosphorylation and it is possible that they may be targets of other KSHV viral proteins such as the latency associated nuclear antigen (LANA) (22).

The biological significance of FKHR retention in the cytoplasm of K1-expressing cells was established by analyzing the functional targets of FKHR. One important target of the forkhead family of transcription factors is the Fas ligand. Specifically, FKHRL1 mediates the transcription of FasL in response to apoptotic stimuli. This activation is mediated through binding of FKHRL1 to the three Forkhead responsive elements

(FHRE) in the FasL promoter. Secreted FasL binds to the Fas receptor on the surface of the cell and establishes a positive feedback loop resulting in cell death. We observed that K1 protects cells from both FKHR-mediated apoptosis and Fas-mediated apoptosis, as analyzed by several different apoptosis assays.

Signaling through the BCR can disrupt the Fas pathway and inhibit apoptosis in both primary and established B cell lines (11, 44). Hinshaw et al. (25) have shown that this protection is mediated upstream of caspase activation. Although Fas-mediated apoptosis has been well studied, FKHR-induced apoptosis is not completely understood. While we did observe that K1 protected cells from FKHR-induced apoptosis, it was to a lesser extent than that seen with Fas-mediated apoptosis. FKHR can transactivate several pro-apoptotic genes but has also been shown to repress gene transcription (43). In addition, the FKHR family can regulate genes directly, by binding to their promoters, or indirectly through interactions with other cellular factors (48, 63). Importantly a FKHR mutant unable to bind DNA to induce cell death, could still induce cell-cycle arrest. Hence, although K1 prevents FKHR from translocating to the nucleus, it may not preclude FKHR's ability to interact with other cellular proteins, and although K1 may be able to repress the FKHR-regulated arm of the Fas-FasL apoptotic pathway, it may not be effective at preventing other types of apoptosis that are regulated by various members of the FKHR family. Conversely, although K1 can prevent FKHR from activating FasL transcription, it is possible that it can inhibit other arms of the Fas-FasL pathway as well, and may explain why K1 protects cells from Fas-mediated apoptosis more effectively.

Our data indicate that the K1 protein can activate the Akt pathway in B lymphocytes and that this activation event is mediated by PI3K. This is consistent with

recent reports indicating that Akt is a target for other transforming viral gene products, simian virus 40 (SV40) large and small t antigens, and Epstein-Barr virus (EBV) LMP1 and LMP2A proteins (17, 47, 53, 59, 60). Similar to K1, the KSHV vGPCR protein has also been shown to tranform cells and target the Akt kinase (3, 37, 39, 49). Although these reports describe Akt as a target, ours is the first to report the FKHR family of transcription factors as the downstream target effectors of a viral transforming protein. Given the role of the FKHR family in activating expression of the pro-apoptotic FasL and Bim-1 genes, and repressing cyclin D1 expression, the FKHR family seem likely candidates for inactivation by viruses to prevent infected cells from undergoing apoptosis.

In summary, we suggest that the role of the KSHV K1 protein in the viral lifecycle is to protect KSHV-infected cells from undergoing premature apoptosis by initiating cell survival signals. Thus, K1's ability to enhance B cell survival may play an important role in the KSHV viral lifecycle.

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CHAPTER THREE

A CRITICAL ROLE FOR ENDOCYTOSIS IN THE REGULATION OF SIGNALING BY THE KSHV K1 PROTEIN

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ABSTRACT

Kaposi's Sarcoma-associated Herpesvirus (KSHV) is a member of the gammaherpesvirus family. KSHV is the etiologic agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). The first open reading frame of the KSHV genome encodes a type 1 transmembrane glycoprotein named K1. K1 is structurally similar to the B-cell receptor (BCR) and its cytoplasmic tail contains an immunoreceptor tyrosine-based activation motif (ITAM) that can activate Syk kinase and the PI3K/Akt pathway. Recent evidence suggests that receptor signaling not only occurs at the cell membrane, but from intracellular compartments as well. We have found that K1 is internalized in a clathrin-dependent manner, and efficient internalization is coupled to its signaling function. Once internalized, K1 traffics from the early endosome to the recycling endosome. Interestingly, blocking K1's activation of Syk and PI3K prevents K1 from internalizing. We have also found that blocking clathrin-mediated endocytosis prevents downstream signaling by K1. These results strongly suggest that internalization of K1 is intimately associated with normal signaling. When K1 internalization was examined in B-lymphocytes, we found that K1 co-internalized with the BCR. Altogether, these results suggest that K1's signaling function is tightly coupled to its internalization.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) is a gammaherpesvirus that was first identified in KS biopsies (5). KSHV has since been found in all epidemiological forms of KS (14). Viral DNA has been consistently isolated in AIDS-associated KS and almost all European/Mediterranean KS (9, 11, 26). KSHV has also been associated with lymphoproliferative diseases, such as PEL and MCD (42), both of which are B-cell in origin. The exact mechanism by which KSHV induces transformation has not yet been completely dissected.

The far left-end of the KSHV genome encodes a 46 Kilodalton (KDa) transmembrane glycoprotein called K1. This position is equivalent to that of the saimiri transformation protein (STP) of herpesvirus saimiri (HVS) (29) and the R1 oncogene of rhesus monkey rhadinovirus (RRV) (10). K1 is expressed in KS lesions, primary effusion lymphoma cells, and multicentric Castleman's disease (1, 16, 20, 35). K1 is structurally similar to the BCR. The cytoplasmic tail contains an immunoreceptor tyrosine-based activation motif (ITAM), which has been shown to be capable of activating a signal profile (18, 22) similar to that activated by the BCR in B-lymphocytes (34). The ITAM is essentially comprised of two SH2 binding motifs. Unlike BCR, K1 is constitutively active, possibly due to oligomerization via conserved, extracellular cysteine residues (18). K1 has been shown to interact with multiple cellular proteins containing SH2 domains, including Lyn, Syk, p85, PLCγ2, RasGAP, Vav and Grb2. This interaction is thought to occur through the phosphorylated SH2 binding motifs that constitute the ITAM in the C-terminus of K1 (21). Furthermore, K1 expression has also been shown to promote the production and secretion of vascular endothelial growth factor (VEGF) in

both epithelial and endothelial cells and to increase matrix metalloproteinase-9 (MMP-9) expression in endothelial cells, all of which is dependent on the SH2 binding motifs in the K1 cytoplasmic tail (48). Transgenic K1 mice develop tumors with features similar to spindle-cell sarcomatoid and malignant plasmablastic lymphoma. Moreover, lymphocytes isolated from these transgenic mice showed constitutive activation of NF-κB and Oct-2, and enhanced Lyn activity (32, 33). Additionally, our lab has previously shown that K1 activates the PI3K/Akt pathway in both B-cells and endothelial cells, protecting cells from apoptosis (43, 47).

Activation of cell surface receptors by specific ligands often results in internalization via clathrin dependent and independent pathways, and internalization of receptors is considered an important mechanism by which cells control the intensity and duration of signal transduction. Recent findings indicate that internalization of receptors can allow signal propagation and amplification due to the high order of regulation of the endosome, using the compartmentalized organization of the endocytic pathway, going beyond the conventional role of receptor/cargo degradation. Some receptors, such as EGF or FGF, can maintain their signaling activities from within intracellular compartments (3, 39).

In this study, we show that K1 is internalized via clathrin-mediated endocytosis, and that K1's ability to signal is coupled to its internalization. We further demonstrate that blocking downstream signaling by K1 prevents its internalization, and that blocking internalization prevents K1 activation of the PI3K/Akt pathway.

MATERIALS AND METHODS

Reagents and Antibodies. LY294002 and Amantadine were purchased from Sigma Chemicals (St. Louis, MO). Piceatannol was purchased from Calbiochem (La Jolla, CA) Anti-FLAG M1 and M2-Cy3 antibodies were purchased from Sigma Chemicals. Anti-Clathrin-HC antibody was purchased from Santa Cruz Biotech. anti-TfR-Alexa 647, anti-IgM-Alexa 647, and anti-Rabbit Alexa-647 were purchased from Molecular Probes, Invitrogen (Carlsbad, CA). Goat anti-mouse IgG, goat anti-mouse HRP and 1-step ABTS were purchased from Pierce (Rockford, IL) anti-Akt (S473), anti-Akt, and anti-Rabbit HRP were purchased from Cell Signaling (Danvers, MA). Anti-EEA1-FITC was purchased from BD Pharmingen (Franklin Lakes, NJ).

cDNAs, cell lines and transfections. pEF-K1$_{WT}$, pEF K1$_{ITAM-}$ have been previously described (43). pEF-K1$_{\Delta C}$ was constructed by deleting the C-terminus of K1 (Figure 1). A cDNA encoding the clathrin hub fragment that contained an amino-terminal T7 epitope (MASMTGGQQMG) was provided by J Trejo (University of North Carolina). Rab-11 GFP was a kind gift from Stephen S. G. Ferguson, University of Western Ontario.

HeLa cells stably expressing the tetracycline-regulatable chimeric transcription factor (tetR-VP16),generously provided by J Trejo (University of North Carolina), were cultured in DMEM supplemented with 10% fetal bovine serum, 4.5 mg/ml glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml G418. Cells were plated at 1.5 x 10^5 or 2.5 x 10^5 cells per well of 12-, or 6-well plates, respectively, and grown overnight. Cells were then transiently transfected with a total of 0.8 or 2.0 µg per well of 12- or 6-well plates, respectively, of plasmids encoding FLAG-tagged K1 wild-type or

mutants and either pcDNA vector, or GFP-fusion constructs using LipofectAMINE Reagent (Invitrogen) according to the manufacturer's instructions.

DG-75 cells, a human B-cell line that is KSHV- and EBV-negative (19), were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin and streptomycin. 40 ug of pEF-K1 wild-type, mutants or empty vector were electroporated in serum free media into 8 x 10^6 DG-75s at 300V and 950μF.

Internalization Assay. To follow internalized receptors, HeLa cells transiently expressing similar amounts of FLAG-K1 wild type or mutants were incubated with the calcium-dependent M1 anti-FLAG antibody for 1 h at 4°C. Under these conditions only receptors present on the cell surface bind antibody. Cells were washed and incubated for various times at 37°C. Surface-bound antibody was then removed by three washes with PBS (Ca^{2+}- and Mg^{2+}-free) containing 0.04% EDTA for 5 minutes at 4°C. Cells were then lysed in Triton lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 3% BSA, and 1% Triton X-100). Sandwich enzyme-linked immunosorbant assay (ELISA) was used in which anti-FLAG antibody was allowed to bind to immobilized goat anti-mouse antibody and was detected by free goat anti-mouse antibody coupled to HRP. The 96-well plates (Greiner Bio-one, Catalog# 655061) were coated with 1 μg/well goat anti-mouse antibody (Pierce) in 0.1 ml of PBS overnight at 4 °C. Each well was next incubated with 0.2 ml of 3% BSA in PBS for 3 h to block nonspecific binding. Lysates were applied to the plates, which were then incubated for 3 h at room temperature and then washed 3x in PBS. Each well was incubated for 1 h with 0.5 μg of HRP-coupled goat anti-mouse antibody in 0.1 ml of PBS containing 3% BSA. After five washes in PBS, 0.2 ml of HRP substrate was added, and incubated in One Step ABTS solution

(Pierce), which contains the substrate for HRP. HRP activity was determined by measuring A of this solution at 405 nm using Fluostar-Optima microplate spectrophotometer (BMG Laboratories.)

Cell Surface Biotinylation and Endocytosis Assay. Transfected HeLa cells grown on 100mm tissue culture plates were incubated with 1.5 mg/ml of sulfosuccinimidyl 2-(biotinamido) ethyl-dithioproprionate (sulfo-NHS-SS-biotin; Pierce Chemical Company) at 4° C for 1 hour, and washed with 15mm glycine in PBS to quench any free sulfo-NHS-SS-biotin. This was followed by several washes with PBS. To measure cell surface biotinylated proteins, cells were then lysed in 0.5 ml of RIPA buffer (20 mm Tris-HCl pH 7.4, with 150 mm NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mm EDTA) containing protease inhibitors. To measure internalized biotinylated proteins, PBS was replaced with complete DMEM medium at 37° C for 10 and 30 minutes. Cells were incubated in two 30-minute washes of glutathione stripping solution (60 mm glutathione and 0.83 mm NaCl, with 0.83 mm NaOH and 1% BSA) at 4° C, which removed all cell surface biotin groups. Remaining biotinylated proteins were sequestered inside cells by endocytosis and were therefore protected from glutathione stripping. Cell extracts were centrifuged to remove cell debris and obtain a detergent soluble supernatant, which was incubated with streptavidin beads (Sigma Chemical Company) to collect bound, biotinylated proteins. These samples were then analyzed by SDS-PAGE and immunoblotted to identify K1-FLAG-fusion proteins.

Immunofluorescence confocal microscopy. HeLa cells plated on Collagen-coated glass bottom dishes (MatTek, Ashland, MA) or DG-75 cells in suspension were preincubated with rabbit polyclonal anti-FLAG-Cy3 antibody for 1 h at 4°C to label the surface

molecules, washed, and then left untreated or treated for various times at 37°C. Cells were fixed and processed for confocal microscopy. Briefly, cells were fixed in 2% paraformaldehyde and permeabilized with 100% methanol. Cells were blocked with 5% normal goat serum. Co-localization of K1 with sub-cellular structures was assessed by incubating permeabilized cells with antibody for 1 h at 25°C, followed by species-specific fluorophore-conjugated secondary antibodies, and then imaged using an Olympus FV500 Confocal Laser Scanning Microscope.

Amantadine and hypertonic treatments. Clathrin pit–mediated endocytosis was inhibited using amantadine (2mM Amantadine in DMEM with 1% BSA) or hypertonic medium (20% sucrose in DMEM with 1% BSA) for 30 minutes as described previously (13, 31) and assayed as indicated in the specific sections.

Western blot assays. To detect Akt phosphorylation, transfected HeLa cells were serum starved for 48 hours; cells were harvested and lysed in RIPA buffer containing protease and phosphatase inhibitors. One hundred µg of each protein sample were subjected to SDS-PAGE and transferred to a nitrocellulose membrane and probed with anti-Akt-Ser473 (Cell Signal). After detection, membranes were stripped and re-probed using Akt (total) antibody (Cell Signal).

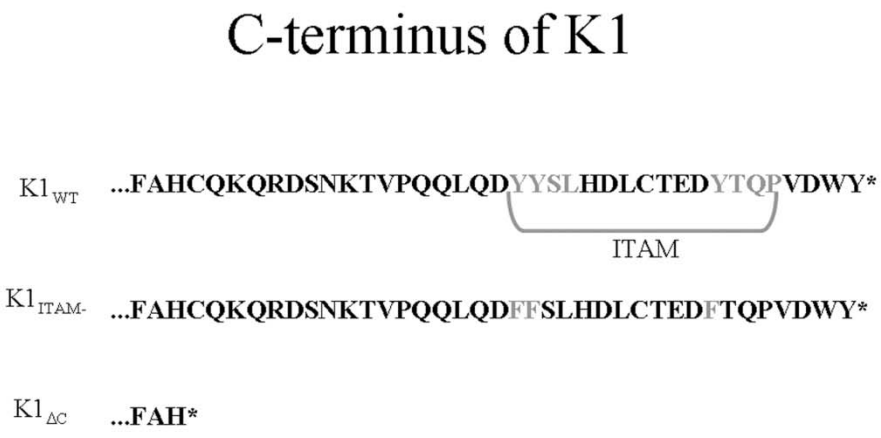
RESULTS

Rate of K1 Internalization. In order to determine how surface expression of K1 is regulated, we first compared internalization rates of wild-type K1, a $K1_{ITAM-}$ mutant in which both tyrosines present in the two SH2 binding motifs have been mutated to phenlyalanines (43), and a mutant of K1 in which the entire carboxyl terminus has been deleted, $K1_{\Delta C}$ (Figure 1A). HeLa cells were transiently transfected with either FLAG-

tagged $K1_{WT}$, FLAG-tagged $K1_{ITAM-}$ or FLAG-tagged $K1_{\Delta C}$ for 48 hours and then labeled with the calcium-dependent M1-FLAG antibody for 1 hr at 4°C. Cells were washed to remove unbound antibody and incubated at 37 °C for various time points. After incubation, the remaining surface bound calcium-sensitive M1-FLAG antibody was stripped using an EDTA/PBS solution. Cells were lysed and the amount of internalized M1-FLAG was quantified by a FLAG ELISA. For $K1_{WT}$, after 10 minutes at 37 °C, ~40% of the receptor had been internalized, appearing to reach a steady-state of internalization. There was a slight increase in internalized K1 to ~60% after 80 minutes at 37 °C (Figure 1B). For the $K1_{ITAM-}$ mutant, ~20% of receptor had been internalized by 10 minutes, and this appeared to be a steady-state level. After 10 minutes, approximately 10% of $K1_{\Delta C}$ surface receptor had been internalized, reaching ~20% internalization after 80 minutes at 37 °C (Figure 1B.) These results suggest that elements in the C-terminus of K1 are involved in internalization. Thus, wild type-K1 internalizes most efficiently, its rate of internalization increasing over time.

To confirm that K1 internalization is not dependent on antibody crosslinking, cell surface biotinylation and internalization of K1 and mutants was analyzed. We used a biotin endocytosis assay that employed the disulfide-linked NHS-SS-biotin, which permits cleavage of any biotin from the cell surface after the 37°C endocytosis step with glutathione (2, 4, 30, 49). Internalization of biotinylated glycoprotein protects the biotin from cleavage. Biotinylated proteins were then immunoprecipitated with streptavidin conjugated beads and subject to SDS-PAGE analysis. Membranes were then probed with

A



B

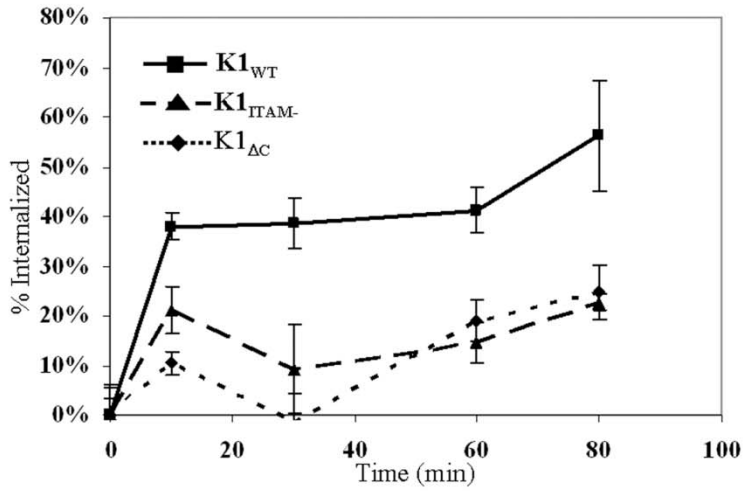
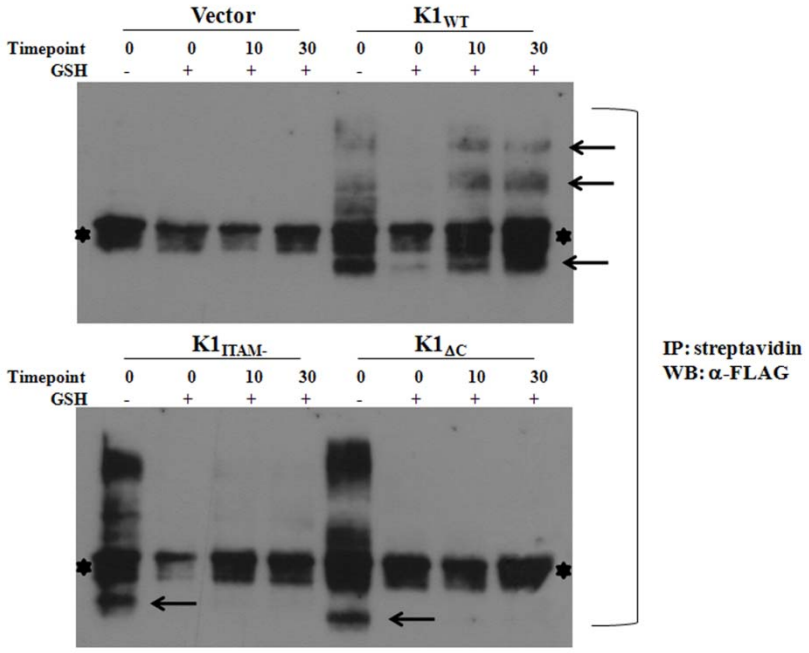


Figure 1. Internalization of K1 in HeLa Cells. (A) Diagram of the amino acids in the C-terminus of K1 and mutants. The ITAM is highlighted. **(B)** Transfected cells were surface labeled with M1 anti-FLAG antibody at 4^{o}C for 1 h, washed and incubated for times indicated at 37^{o}C. Antibody remaining on the surface was stripped off and cells were lysed. Internalized antibody was quantified by ELISA. The data are expressed as a percentage of the initial amount of antibody bound to the cell surface at 0 minutes at 4^{o}C.

C



D

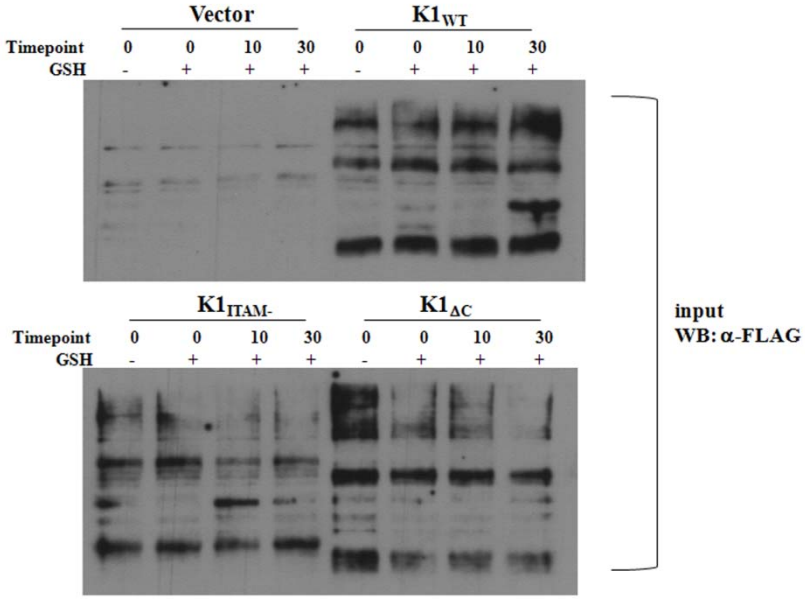


Figure 1.cont'd. (C) Endocytosis of K1 in a biotin endocytosis assay. HeLa cells were transfected with K1 and mutant expression plasmids as indicated above each gel. The samples were processed for the biotinylation endocytosis assay as described in the methods section. Biotinylated proteins were internalized by transferring the cells to 37° C for 10 and 30 minutes. The + and – symbols indicate glutathione (GSH) treatment. Cells were then lysed and all biotinylated proteins immunoprecipitated using streptavidin beads. FLAG-K1 was detected in cell surface and intracellular pools by immunoblotting. **(D)** Same samples as in C, representing 1% of input sample before immunoprecipitation.

antibody specific to the N-terminal FLAG epitopes on each construct. The 0 min- lane shows total cell surface biotinylation of K1 proteins before the glutathione cleavage step. The 0 min+ lane indicates that the cells were not returned to 37°C and were instead immediately treated with glutathione; the absence of K1-specific bands demonstrated that the cleavage step removed all remaining cell surface biotin to an undetectable level. For the 10 min+ and 30 min+, cells were incubated at 37°C for the indicated time before the cleavage step. Thus, if K1were internalized, a band would be detectable in these lanes. As can be seen in Figure 1C, $K1_{WT}$ internalization is detectable by 10 minutes after incubation at 37°C, and continues at 30 minutes at 37°C. However, for the two mutants, $K1_{ITAM-}$ and $K1_{\Delta C}$, no specific bands appear at the times tested, indicating that neither of these two mutants were protected from cleavage by glutathione, and were therefore not endocytosed. The banding pattern seen for the K1 constructs is typical, showing a major band at 46KDa, and larger bands running at 100, 150, and 200KDa, most likely due to oligomerization through extracellular disulfide bonds (17, 18, 23), indicated by arrows. The asterisk denotes non-specific bands. Input levels of biotinylated proteins are also shown (Figure 1D). Before immunoprecipitations were performed, a fraction of the lysate from each sample was removed and subjected to SDS-PAGE analysis. Membranes were probed with same antibody as in Figure 1C.

Confocal microscopy analysis of HeLa cells transiently transfected with the WT and mutant K1 constructs support our ELISA data. Cells transfected with FLAG-tagged $K1_{WT}$, FLAG-tagged $K1_{ITAM-}$ or FLAG-tagged $K1_{\Delta C}$ were labeled with an anti-FLAG-Cy3 antibody, washed, and incubated for various times at 37°C. After incubation, cells were fixed and analyzed by confocal microscopy. After 20 minutes incubation, $K1_{WT}$

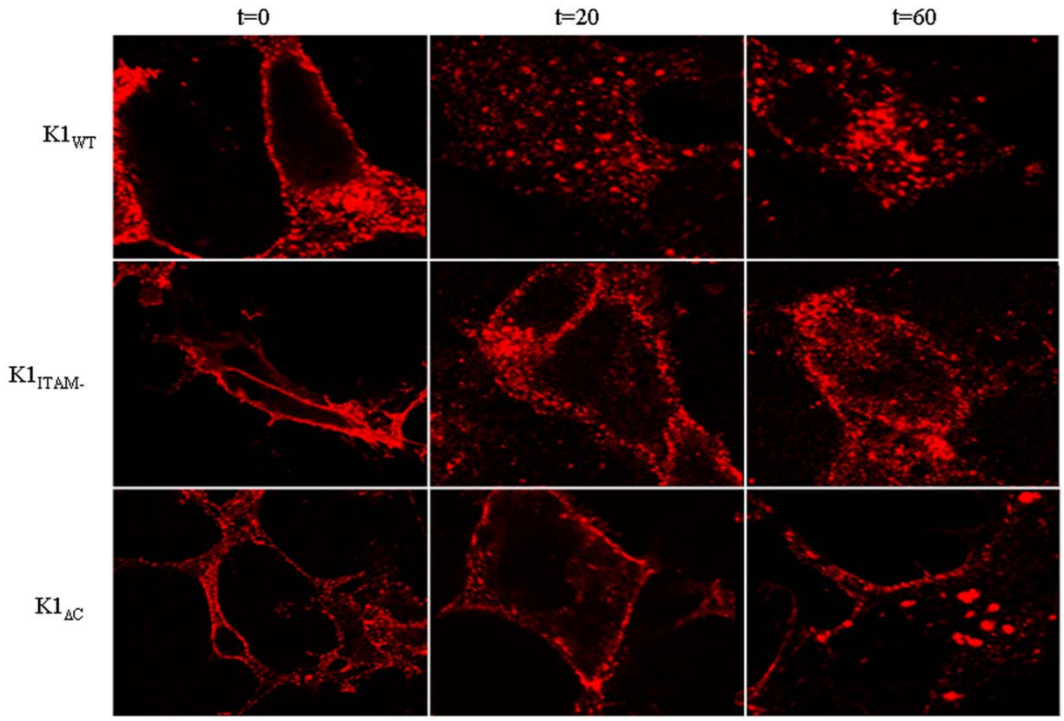


Figure 2. **Confocal analysis of K1 internalization.** Cellular localization of K1 after surface labeling of K1-FLAG constructs. Cells were labeled with anti-FLAG-Cy3 at 4°C for 1h, and then transferred to 37°C for times indicated. Cells were fixed and analyzed by confocal microscopy. K1 expression constructs used are described in Figure 1.

localized predominantly to endocytic-like structures (Figure 2). In contrast, $K1_{ITAM-}$ and $K1_{\Delta C}$ mutants were maintained predominantly at the surface at both 20 minutes and 60 minutes suggesting that their internalization is severely compromised.

We next determined whether K1 was recycled after being endocytosed. EEA1 served as a marker of early endosomes, and Rab11 was used as a marker for recycling endosomes. HeLa cells were labeled for K1 surface expression as described above and incubated for 20 minutes at 37°C. Permeablized cells were labeled with anti-EEA1-FITC. EEA1, is a Rab5 effector protein associated with the cytoplasmic side of early endosomes (6). As can be seen in Figure 3A, wild-type K1 co-localizes with EEA1, indicating K1 traffics to the early endosome. While the majority of $K1_{ITAM-}$ and $K1_{\Delta C}$ remains on the surface of the cells, what little does internalize, appears to co-localize with EEA1. We also analyzed K1 trafficking without pre-labeling with a Flag antibody. Transfected HeLa cells were placed on ice for 10 min, and then moved to 37°C for 20 minutes. Cells were permeablized and stained with anti-FLAG-Cy3 and anti-EEA1-FITC antibodies, and analyzed by confocal microscopy. Similar to pre-labeled cells, wild-type K1 appears to co-localize with EEA1 (Figure 3B), with little or no co-localization of EEA1 with $K1_{ITAM-}$ and $K1_{\Delta C}$.

We also co-expressed K1 with Rab11-GFP. Rab11 has been shown to coordinate traffic through the recycling endosome (45). Our confocal analysis revealed that $K1_{WT}$ appears to traffic to the recycling endosome, as indicated by co-localization with Rab-11-GFP after incubating at 37°C for 90 minutes. Conversely, $K1_{ITAM-}$ and $K1_{\Delta C}$ do not appear to co-localize with Rab-11 (Figure 3C). K1 trafficking to the recycling endosome was examined without pre-labeling, as well. As can be seen in figure 3D, $K1_{WT}$ co-

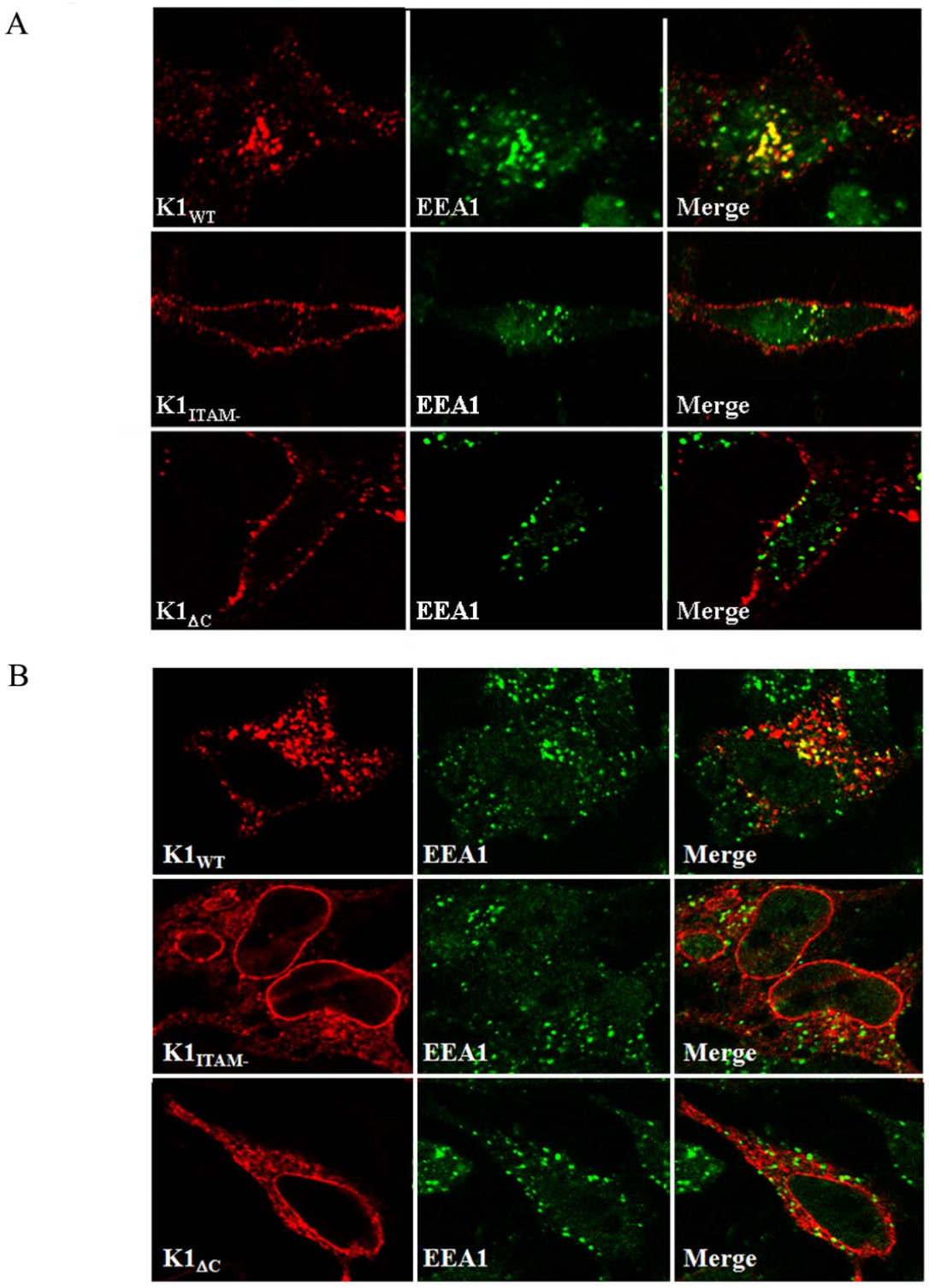
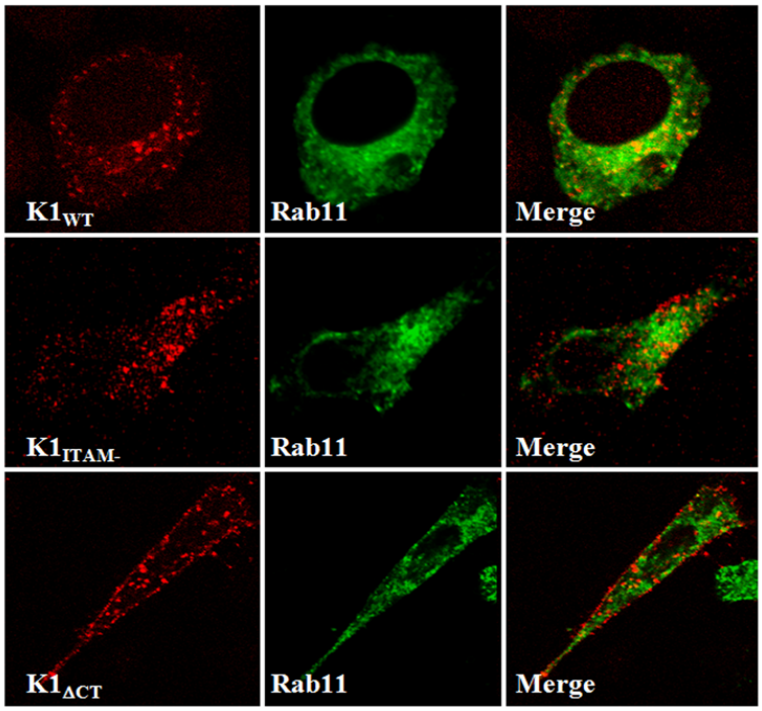


Figure 3. Sub-cellular trafficking of K1. (A) K1 expressing HeLa cells were incubated with anti-FLAG-CY3 for 1h at 4^{o}C. Bound receptors were allowed to internalize for 20 minutes at 37^{o}C and subsequently labeled with FITC-labeled anti-EEA1 antibody. **(B)** K1 expressing HeLa cells were fixed, permeablized and labeled with anti-FLAG-CY3 and FITC-labeled anti-EEA1 antibody.

C



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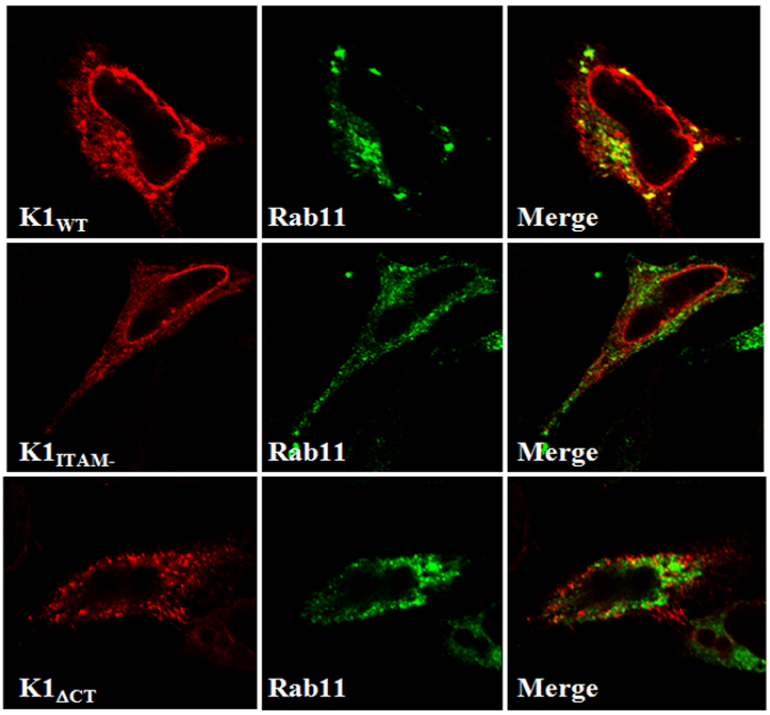


Figure 3 cont'd (C) HeLa cells were co-transfected with K1 expression vectors and a Rab11-GFP expression vector. Cells were surface labeled with anti-FLAG-CY4 for 1h at 4°C. Cells were incubated at 37°C for 90 minutes. Cells were fixed and imaged by confocal microscopy**. (D)** HeLa cells co-transfected with K1 constructs and Rab11-GFP were fixed, permeablized and labeled with anti-FLAG-CY3 and analyzed by confocal microscopy.

localizes with Rab11 in contrast to the two K1 mutants.

K1 is internalized in a clathrin-dependent manner. We next investigated whether K1 is internalized through a clathrin-dependent pathway. We first analyzed co-localization of WT K1 and the clathrin heavy-chain. WT FLAG-K1 transfected HeLa cells were incubated with anti-FLAG-Cy3 at 4°C, so that only K1 on the cell surface is labeled. Cells were placed at 37°C for 5 minutes, fixed, permeabilized, and labeled with anti-Clathrin-FITC antibody, which stains the clathrin heavy chain. Cells were examined by confocal microscopy. As can be seen in Figure 4A, K1 appears to co-localize with clathrin. We next looked at the effect of a dominant-negative clathrin hub. Hub is the C-terminal third of the clathrin heavy chain (25) and has been shown to act as a dominant-negative clathrin inhibitor by competing for light chain binding (24). Cells were transiently transfected as above with the addition of an expression vector encoding the clathrin hub fragment with an amino-terminal T7-epitope. Cells were labeled with an anti-FLAG-Cy3 at 4°C and moved to 37°C for 20 minutes. Cells were then fixed and permeabilized. Cells expressing the clathrin hub mutant were identified by co-staining for the T7 epitope. Antibody to the T7 epitope labeled the entire cytoplasm as previously described (44). We found that cells which express the clathrin hub showed decreased internalization of K1, with K1 primarily localized to the cytoplasmic membrane after 20 minutes at 37°C (Figure 4B); further supporting the hypothesis that K1 is internalized in a clathrin-dependent manner.

We next examined the effect of clathrin inhibitors on K1 internalization. Amantadine is a drug that inhibits clathrin-coated pit invagination at the plasma

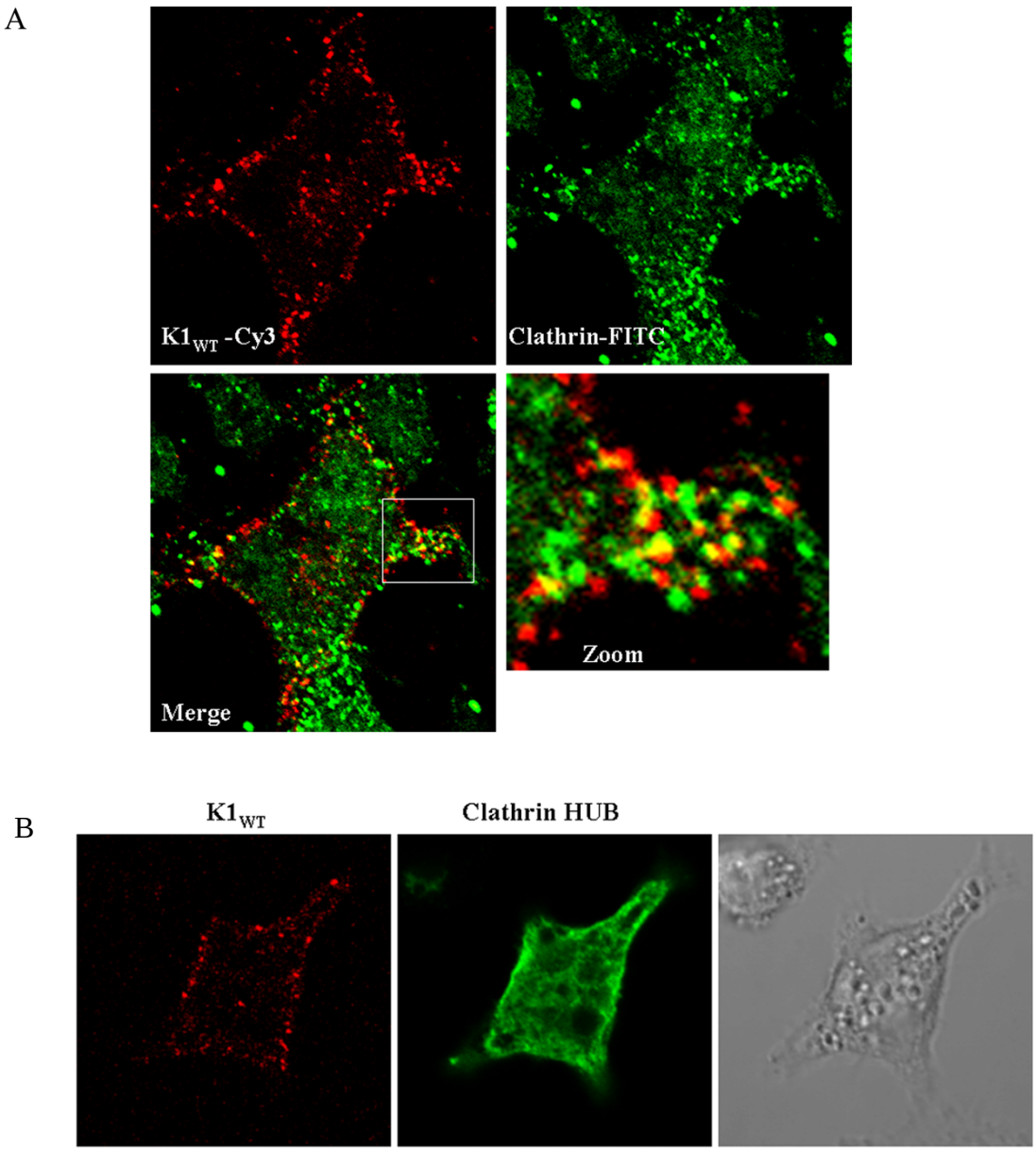


Figure 4. **K1 is internalized in a clathrin-dependent manner. (A)** Co-localization of $K1_{WT}$ with Clathrin-HC. K1 expressing cells were surface labeled with anti-FLAG-Cy3 for 1h at 4°C. After incubation at 37°C for 5 minutes, cells were fixed and immunostained for clathrin-HC. Bound antibodies were visualized with the use of secondary antibody conjugated to FITC. The boxed region indicates magnified area. **(B)** HeLa cells were co-transfected with K1 and T7-Hub. Cells were labeled with an anti-FLAG-Cy3 at 4°C and incubated at 37°C for 20 minutes, then processed as described. Cells expressing the clathrin hub mutant were identified by co-staining for the T7 epitope.

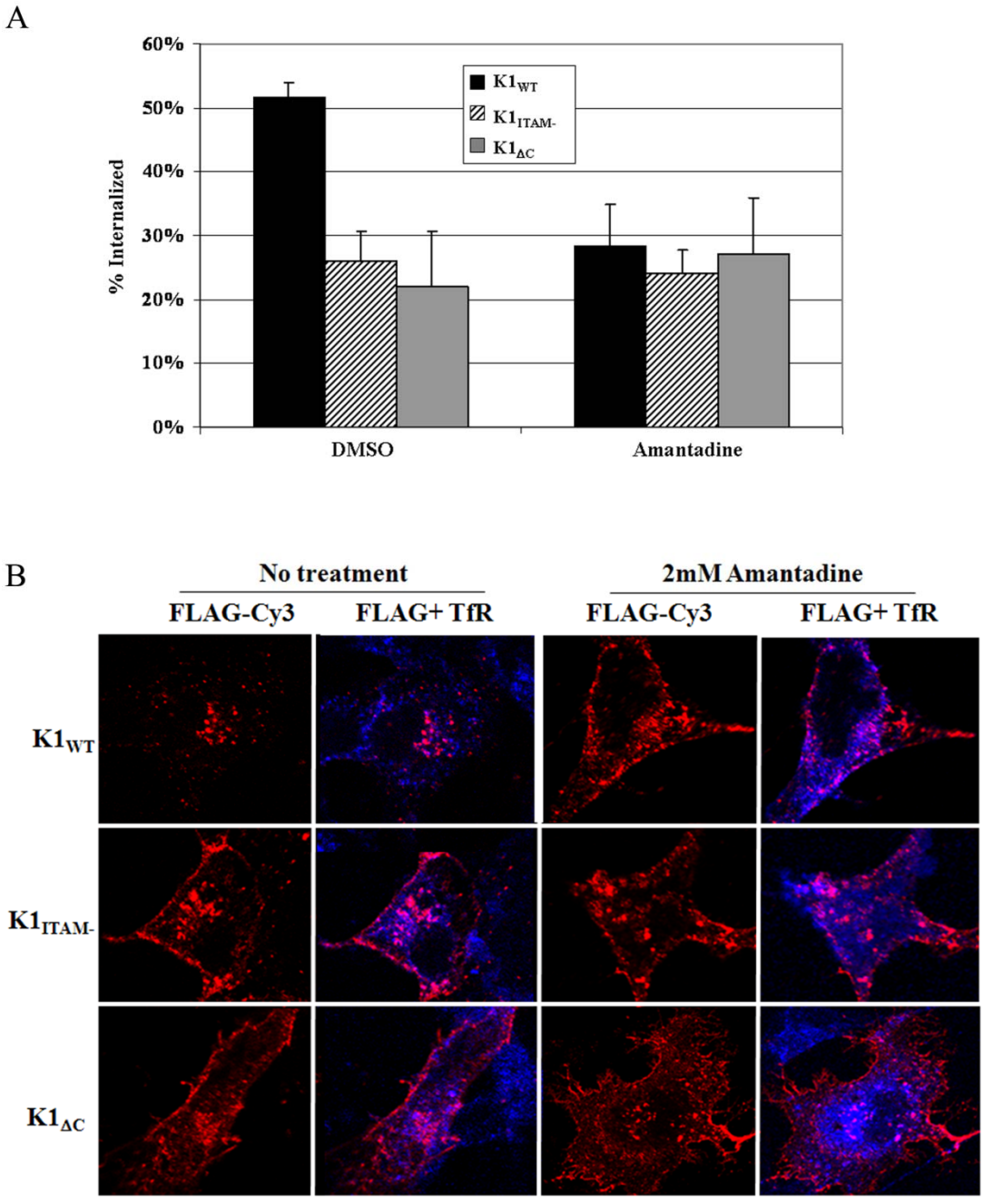


Figure 5. **Amantadine inhibits K1 internalization. (A)** Transfected HeLa cells were surface labeled with M1 anti-FLAG, and surface receptors were allowed to internalize for 20 minutes in the presence or absence of drug. Amount of internalized antibody was quantified by ELISA. The data are expressed as a ratio of the initial amount of antibody bound to the cell surface at 0 minutes at 4^{o}C, to the amount of antibody bound in the presence of drug. **(B)** K1 expressing cells were surface labeled with anti-FLAG-Cy3 and anti-TfR-Alexa647 for 1h at 4^{o}C. Surface receptors were allowed to internalize at 37^{o}C for 20 minutes in the presence of the drug. Cells were fixed and analyzed by confocal microscopy.

membrane (31). Cells transiently transfected with either FLAG-tagged $K1_{WT}$, FLAG-tagged $K1_{ITAM-}$ or FLAG-tagged $K1_{\Delta C}$ were stained as described above and incubated in the presence or absence of amantadine for 20 minutes at 37°C. Cells were then assayed for internalized K1 protein via ELISA. In wild-type K1 cells, internalization was substantially inhibited by amantadine (Figure 5A), while both mutants were less affected by clathrin inhibition. Conversely, K1 internalization was not inhibited by the caveolin inhibitor, filipin (data not shown). Shown in Figure 5B is confocal data supporting inhibition of K1 internalization by amantadine. K1 expressing cells were stained as described above. As a positive control, we also labeled the transferrin receptor (TfR) using anti-TfR Alexa 647 antibody (Molecular Probes). The transferrin receptor has been well studied and constitutively cycles between the plasma membrane, early endosomes, and recycling endosomes, and has serves as a classic system for the study of clathrin-mediated endocytosis (8). Amantadine prevented the majority of K1 protein from internalizing, as well as preventing TfR internalization (Figure 5B).

K1 endocytosis and signaling are coupled. Recent work has shown regulation of receptor signaling as a function of receptor internalization. Subcellular localization can determine which effector molecules couple to activated receptors and the relative strengths of different signaling pathways. To that end, we wanted to determine if known signaling molecules activated by K1 are co-internalized and/or co-localized intracellularly. We examined whether K1 co-localized with the p85 subunit of PI3K. Cells were labeled as above and incubated at 37°C for 5 minutes. Cells were immediately placed on ice, rinsed with cold PBS and fixed. After permeabilization, cells were incubated with anti-p85 antibodies and examined by confocal microscopy. $K1_{WT}$

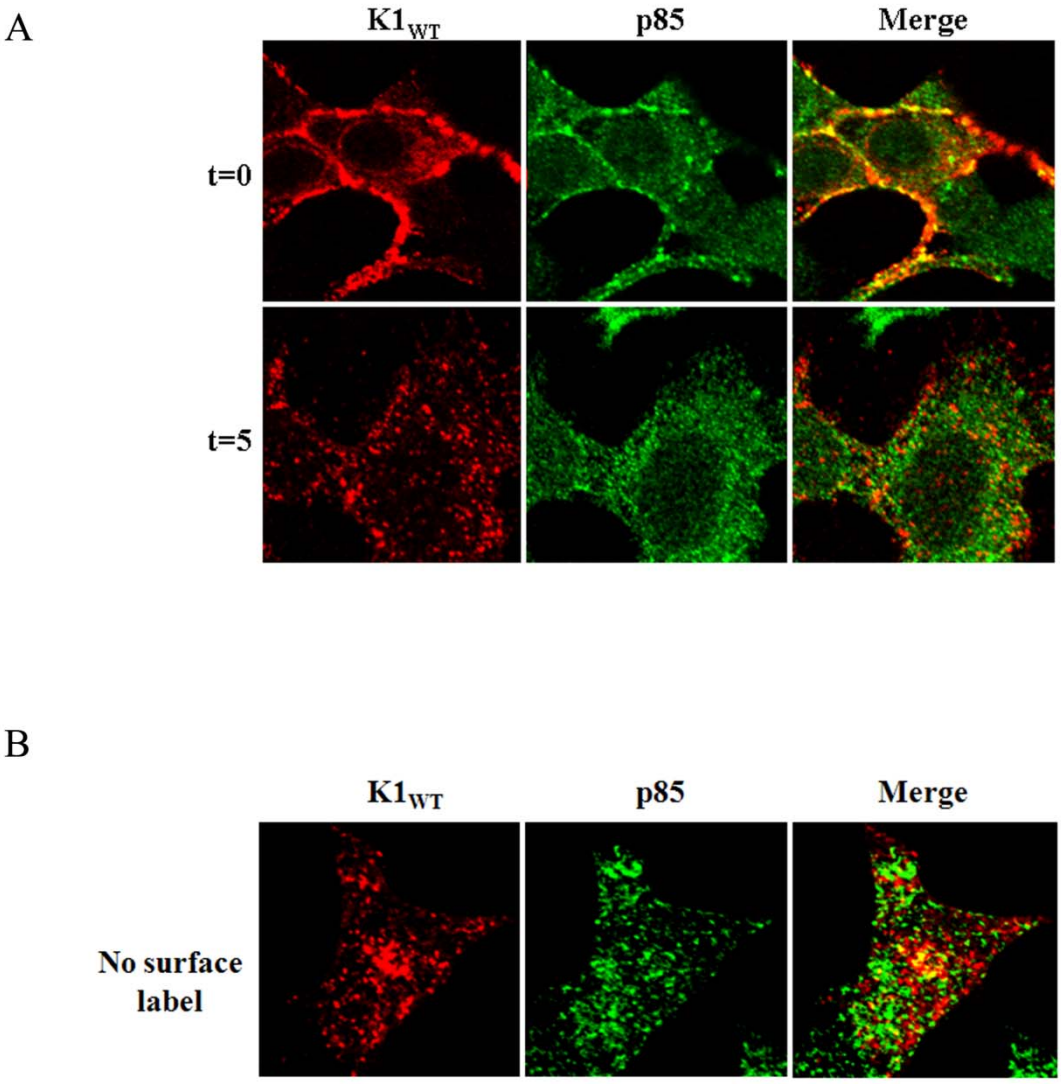


Figure 6. Intracellular distribution of K1 and PI3K. (A) $K1_{WT}$ transfected HeLa cells were surface labeled with anti-FLAG Cy3 incubated at 37 ^{o}C for 5 minutes, immediately placed on ice and fixed. (B) $K1_{WT}$ transfected HeLa cells were fixed, permeabilized and stained with anti-FLAG-Cy3 and anti-p85 primary antibody followed by a FITC-conjugated secondary antibody. Cells were analyzed by confocal microscopy.

and the p85 subunit of PI3K appear to co-localize in discrete punctate-like structures (Figure 6A). This suggests that K1 is endocytosed either with PI3K, or the two are brought together rapidly after K1 internalization. We also analyzed K1 co-localization with p85, without pre-labeling K1 surface molecules. Transfected HeLa cells were placed on ice for 10 min, then moved to 37°C for 5 minutes. Cells were permeablized and stained for FLAG-K1 and p85. As can be seen in Figure 6C, K1 and p85 show considerable co-localization, further supporting the interaction of K1 and PI3K intracellularly.

To further delineate the relationship between signaling and internalization, we utilized specific chemical inhibitors of kinases activated by K1 such as PI3K and Syk, and determined their affect on K1 internalization, by both ELISA and confocal microscopy. LY294002 is an inhibitor of PI3K and piceatannol is a Syk kinase inhibitor. HeLa cells were transfected with K1 and K1 mutant expression vectors, and processed as above. Additionally, cell-surface expressed TfR was labeled using anti-TfR Alexa 647 antibody for confocal studies. LY294002 was added during subsequent 37°C incubations. As determined by ELISA, LY294002 inhibited wild-type K1 internalization by over two-fold after 20 minutes, but had little or no effect on the $K1_{ITAM-}$ or $K1_{\Delta C}$ proteins (Figure 7A). This is further corroborated by confocal data showing little or no wild-type K1 internalization when cells were treated with LY294002 (Figure 7B). In contrast, the TfR is internalized in an efficient manner.

K1 has also been shown to activate Syk kinase (21). We next tested whether piceatannol, an inhibitor of Syk kinase, affected K1 endocytosis. We found that piceatannol also inhibits K1 internalization, although not to the same extent as seen with

A

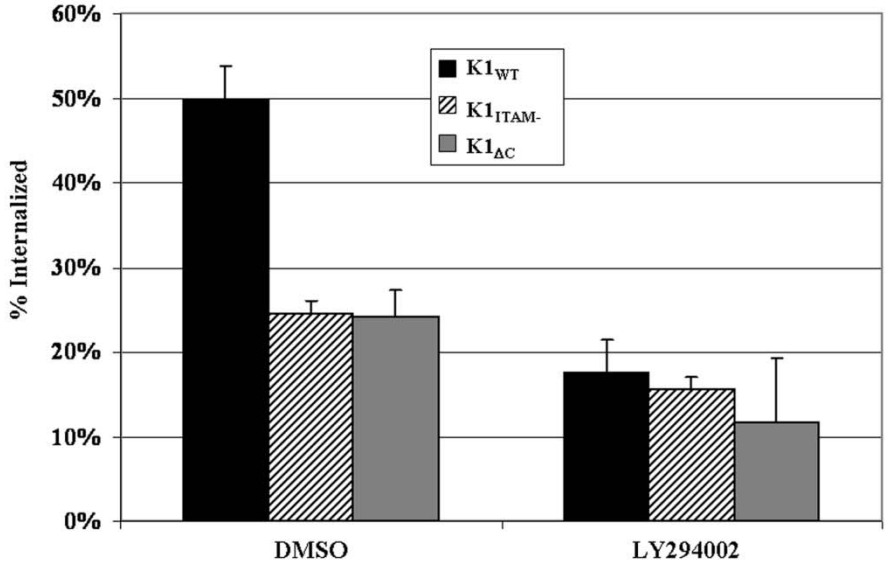


Figure 7. Inhibition of K1 internalization by LY294002. (A) K1 internalization was measured in the presence or absence of LY294002. Amount of internalized receptor was quantified by ELISA. The data are expressed as a ratio of the initial amount of antibody bound to the cell surface at 0 minutes at 4^{o}C, to the amount of antibody bound in the presence of drug, after incubating cells at 37^{o}C for 20 minutes.

B

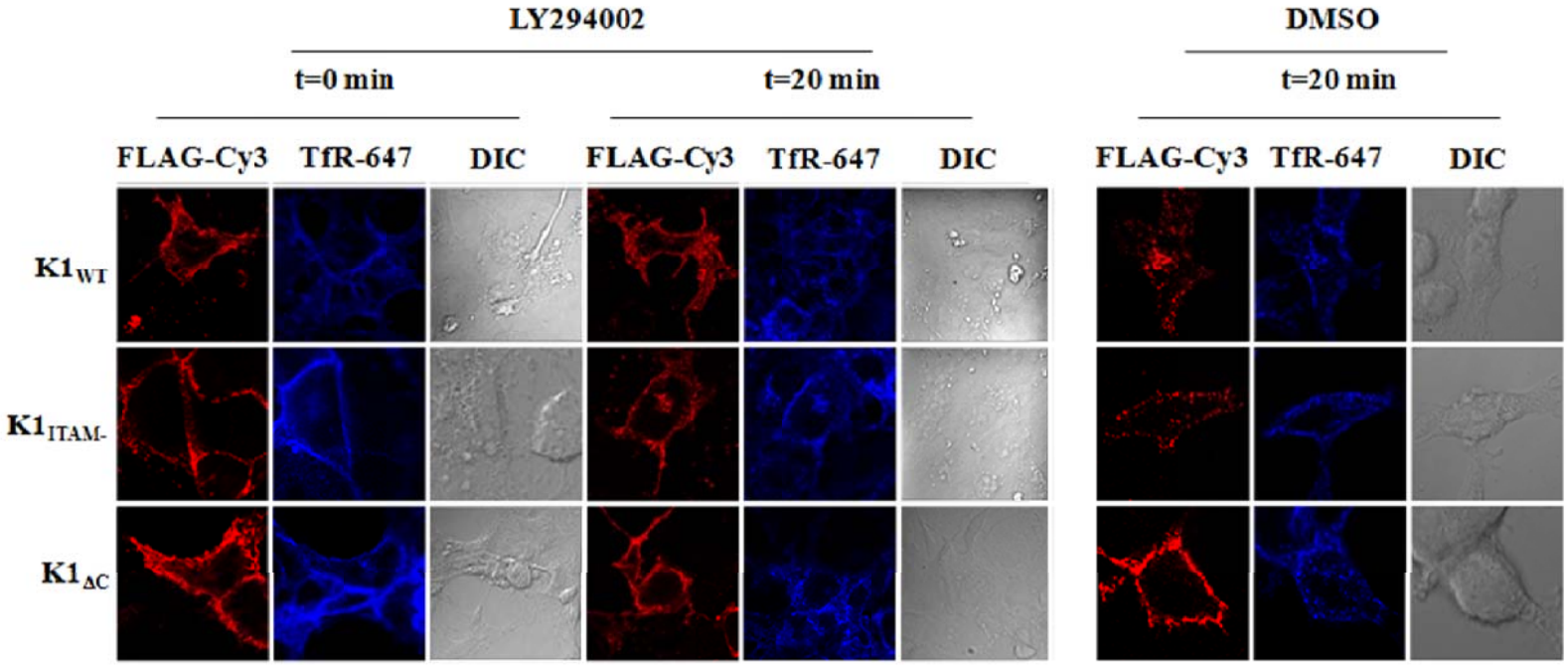


Figure 7 cont'd. **(B)** K1 internalization in the presence or absence of LY294002 as analyzed by confocal microscopy.

LY294002 (Figure 8A) resulting in approximately 50% decrease after cells were incubated for 20 minutes at 37^{o}C in the presence of the drug. Confocal data also supported this, showing that the majority of K1 remains on the surface in the presence of piceatannol (Figure 8B). The two K1 mutants, $K1_{ITAM-}$ or $K1_{\Delta C,}$ were retained on the surface, regardless of drug treatment. It is noted that the staining pattern of K1 surface expression in piceatannol treated cells is qualitatively different than in other confocal experiments. It is possibly that piceatannol disrupts the trafficking of K1 to the cell surface. It has been shown that treatment of cells with piceatannol interrupts exocytosis of molecules (27, 28, 38), which could explain the differential staining pattern. This phenomenon is currently under investigation.

Inhibition of endocytosis prevents signaling by K1. To examine whether K1 endocytosis and trafficking are important for controlling the signaling pathways and cellular responses to K1, we inhibited endocytosis by treating cells with amantadine or hypertonic media. Hypertonic media has been shown to inhibit clathrin-mediated endocytosis (13). HeLa cells were transiently transfected as above. 24 hours post-transfection, cells were serum starved for an additional 24 hours. Cells were pretreated for 30 minutes in fresh media containing 2mM amantadine or 20% sucrose. Cells were harvested and lysates were separated by SDS/PAGE, blotted to membranes, and probed with anti-phospho-Akt (S473). As shown in Figure 9, expression of WT K1 increased Akt phosphorylation in these cells, while the $K1_{ITAM-}$ and $K1_{\Delta C}$ did not induce Akt phosphorylation. Treatment with the endocytosis inhibitors, amantadine and sucrose, dramatically decreased Akt phosphorylation in wild-type K1 expressing cells (Figure 9).

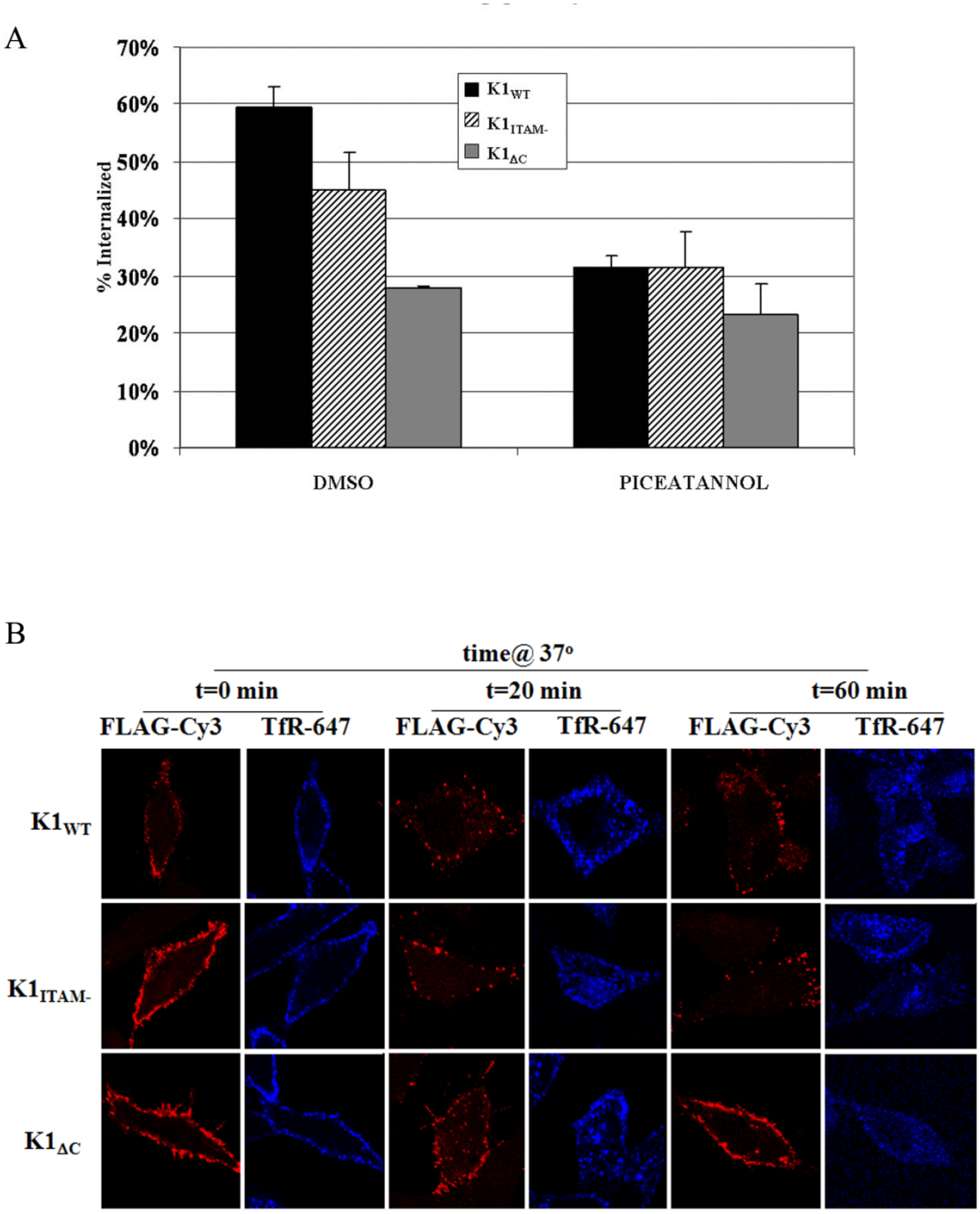


Figure 8. Treatment of cells with piceatannol inhibits K1 internalization. **(A)** Cells were incubated in the presence of 15ug/mL piceatannol. Amount of internalized receptor was quantified by ELISA. The data are expressed as a fraction of the initial amount of antibody bound to the cell surface at 0 minutes at 4°C**. (B)** Confocal microscopy analysis of K1 internalization in the presence of piceatannol.

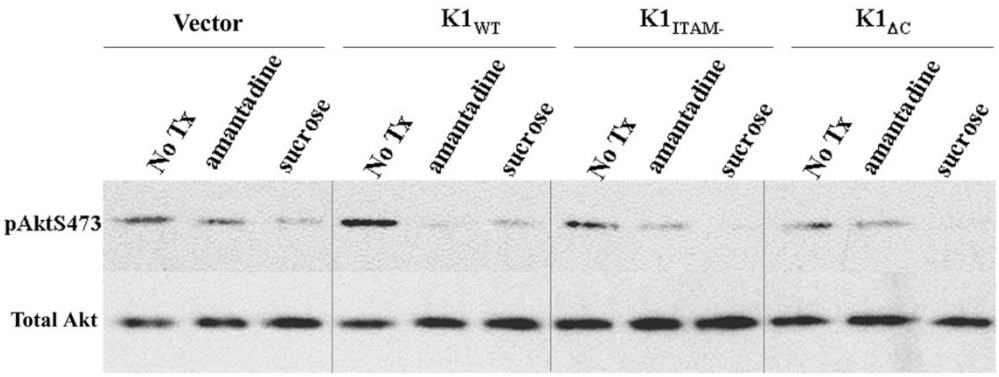
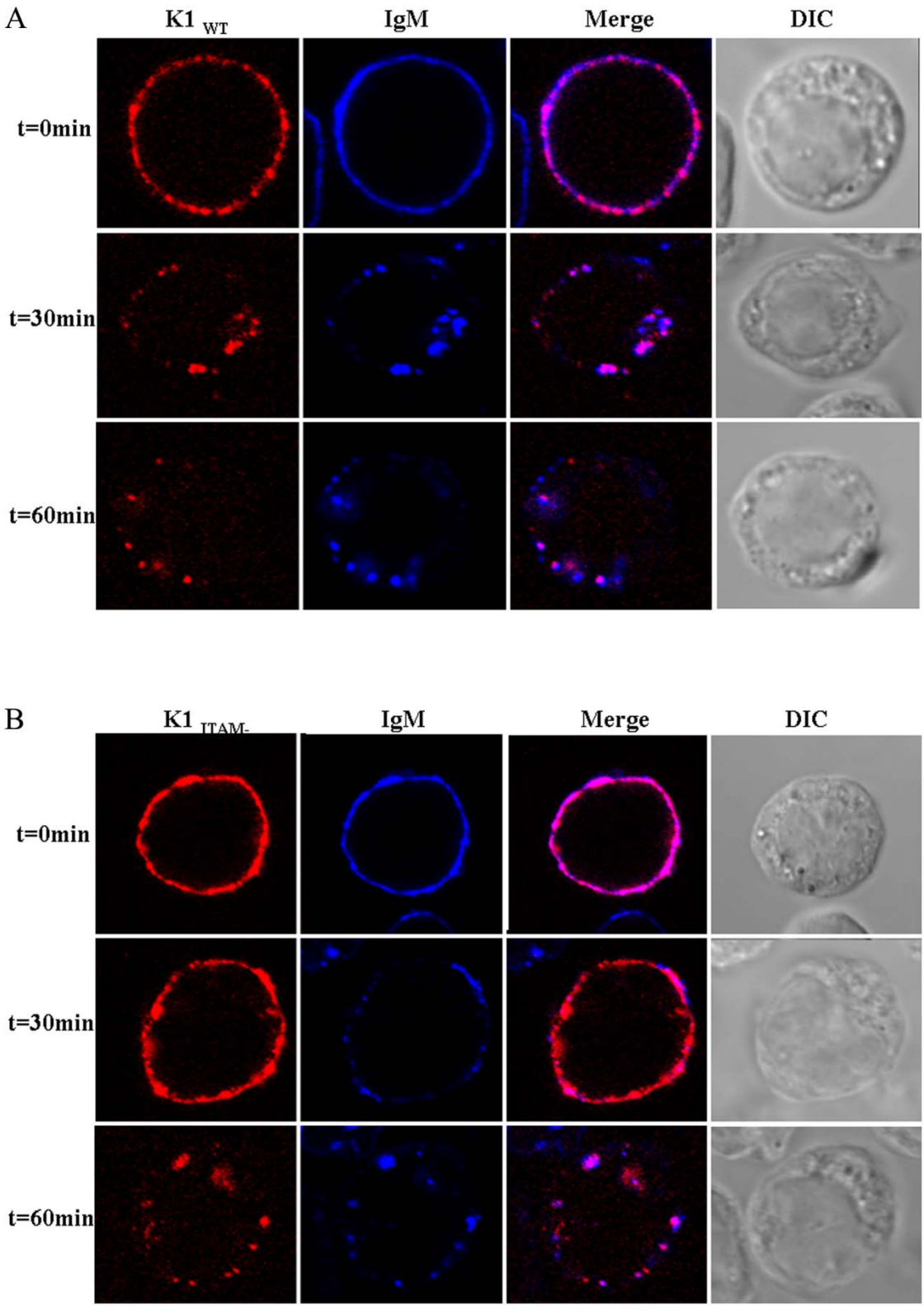


Figure 9. **Inhibition of clathrin-mediated endocytosis prevents K1 signaling.** K1 expressing cells were treated with amantadine or hypertonic media to inhibit clathrin. Equal amounts of total cellular protein were immunoblotted with antibodies to Akt. The membranes were first probed with phospho-specific antibody to Akt S473(top panel), stripped and probed with antibody that recognizes total Akt levels.

K1 internalization in B-cells. We next analyzed K1 internalization in DG75 B-cells, which are KSHV negative. Since KSHV infects B lymphocytes, we wanted to determine if K1 co-internalized with the B cell receptor (BCR). Cells were electroporated with K1 and mutant expression vectors as described in the Methods section. 24 hours post-transfection, cells were surface labeled for K1 using anti-FLAG Cy3 antibody and IgM using anti-IgM Alexa 647 antibody. After rinsing away unbound antibody, cells were incubated at 37°C for indicated times, fixed and analyzed by confocal microscopy. As can be seen in Figure 10A, K1 and IgM co-localize and internalize together. Interestingly, after 30 minutes at 37°C, the $K1_{ITAM-}$ and $K1_{\Delta C}$ mutants (Figure 10B and C) remain bound together with BCR on the surface of the cells and do not internalize in contrast to wild-type K1 and IgM which have been completely internalized at 30 minutes. By 60 minutes, the majority of $K1_{\Delta C}$ and IgM remain on the surface but the majority of $K1_{ITAM-}$ and IgM were internalized. These results show that $K1_{WT}$ co-internalizes with the BCR.

DISCUSSION

It is well established that K1 is expressed on the surface of cells and activates signaling pathways, including the PI3K/Akt pathway, through its C-terminal SH2 binding motifs that encode the ITAM (18, 22, 43). In order to further elucidate the mechanism of K1 signaling, we have examined how its signaling function at the plasma membrane is regulated. In this study we demonstrate that for K1, endocytosis is contingent upon signaling. The two mutants, $K1_{ITAM-}$ and $K1_{\Delta C}$, show decreased internalization as compared to wild-type K1. This suggests that elements in the C-terminus of K1 are required for efficient internalization. Inhibiting K1 activation of PI3K and Syk kinase


A
K1 WT
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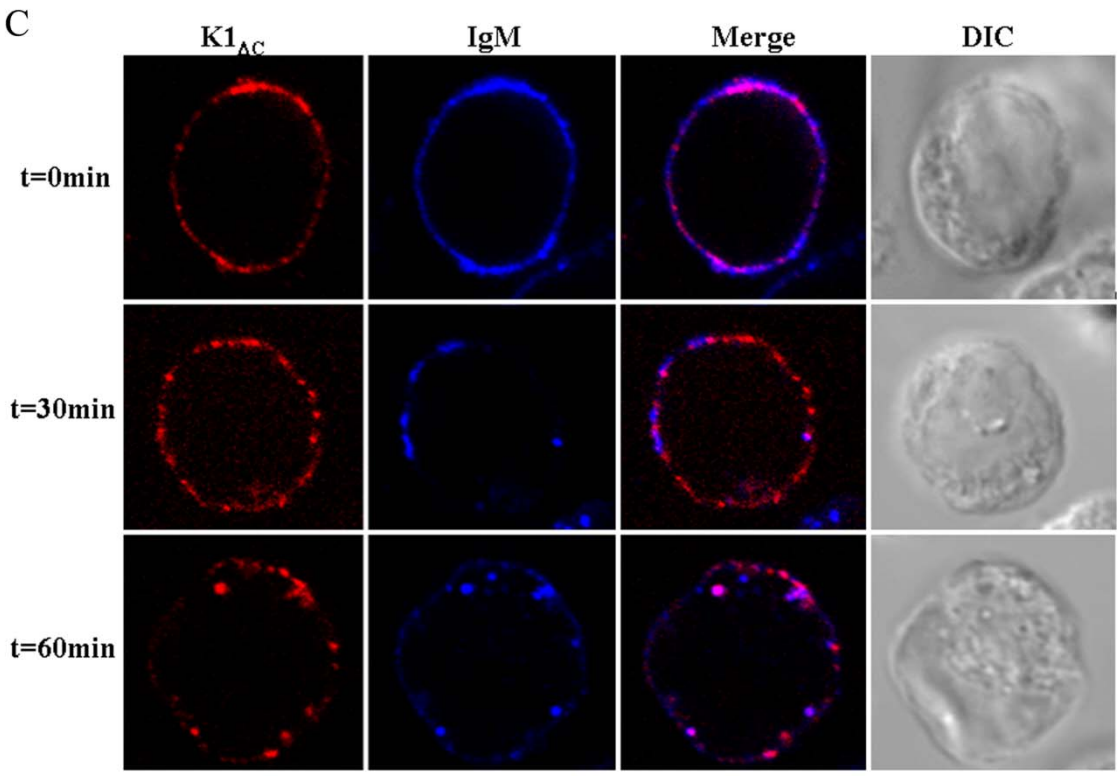

Figure 10. K1 co-internalizes with IgM. DG-75 cells were transfected with $K1_{WT}$ (panel A), $K1_{ITAM-}$ (panel B), or $K1_{\Delta C}$ (panel C) expression vectors. 24 hours post-transfection, cells were surface labeled with anti-FLAG-Cy3 (red) and anti-IgM-647 (blue) antibodies at 4°C for 30 minutes. Cells were transferred to 37°C for times indicated, fixed and viewed by confocal microscopy. Co-localization is indicated by magenta color.

using LY294002 and piceatannol, respectively, resulted in decreased endocytosis of $K1_{WT}$, with little effect on the two mutants tested, further supporting the role of signaling in the control of K1 surface expression. We have also observed the reciprocal result; inhibiting internalization prevents efficient down-stream signaling by K1.

In order to determine the mechanisms controlling K1 internalization, we analyzed the trafficking pattern of surface labeled $K1_{WT}$ and mutants. Perturbing clathrin-dependent endocytosis markedly decreased the rate at which K1 was internalized. We could inhibit K1 internalization by using a dominant-negative clathrin, Hub, further supporting our data that K1 is internalized via clathrin. Following internalization, K1 was present in both early endosomes since it co-localized with EEA1, and in recycling endosomes since it co-localized with Rab11. Like K1, other viral glycoproteins such as the Us28 glycoprotein of CMV (12) show ligand-independent constitutive internalization and recycling. Constitutive recycling may provide a convenient method for the cell to regulate surface receptor numbers. If a rapid decrease in surface receptors is needed, the internalization rate could be increased and/or recycling decreased, and vice versa.

In addition to receptor down-regulation, internalization of receptor subunits also serves to modulate receptor signaling pathways. Our lab has previously shown that K1 can activate Akt via a PI3K-dependent pathway (43). Here we demonstrate that the ability of K1 to activate Akt kinase was suppressed in cells in which endocytosis had been inhibited (Figure 9). The suppression of down-stream signaling has been shown for cellular receptors as well. Using an internalization defective cell line it was shown that EGFR-mediated tyrosine phosphorylation and mitogen-activated protein kinase activation were both attenuated in internalization defective cells (46). Further, correct trafficking of

EGFR is required to establish specific signaling pathways. Membrane trafficking regulates the signal transduction events of EGFR. There is also evidence that receptor tyrosine kinases signal from endosomes. It has been demonstrated that the internalized EGFR can still signal (7). EGFR remains bound to EGF within the endosomal compartment; EGF-stimulated EGFRs preserve their dimerization and their kinase activity within endosomes (41, 50). Receptor signaling from endosomes might allow for spatial and temporal regulation of signal transduction. Signal transduction could be compartmentalized at the plasma membrane and on endosomes. Compartmentalization would provide high flexibility to an otherwise limited number of signaling cascades to transduce specific signals from multiple signaling cues. There is, in fact, accumulating evidence that signaling from endosomes could be used as a means to compartmentalize signal transduction (37, 40).

The co-localization of K1 and the p85 subunit of PI3K are consistent with models of PI3K function, in which PI3K, a principally cytoplasmic protein, is recruited to membranes quantitatively in response to receptor activation. In cells in which endocytosis is inhibited, the p85 subunit of PI3K is hypophosphorylated (46). The endosomes are highly enriched for PI3K activity. It has been found that when the PDGF receptor is stimulated, the receptor is found in isolated-clathrin coated pits. The kinase activity remains associated with receptors as they internalize into endosomes and move from the plasma membrane to the TGN (15).

Lee at al. (21) have shown that K1 interacts with different signaling proteins through its C-terminal tyrosine residues with different affinities. It is easy to imagine that this differential phosphorylation of the tyrosine residues, in differing locations in the cell

i.e. plasma membrane versus endocytic vesicles, allows for K1 to interact with various cellular proteins. Indeed, several receptors can transmit signals from endocytic compartments, and these signals can be qualitatively different from those initiated at the plasma membrane. For example, TrkA promotes NGF-mediated cell survival at the cell surface, whereas it induces differentiation when internalized (51). Internalization could couple activation with down-regulation, which allows for precise temporal control of signaling by limiting the lifetime of the activated signaling components.

The fact that K1 co-internalizes with the BCR in B-cells suggests a physiologically important role for K1 internalization. It is plausible that K1 may function to scavenge the BCR from the surface of newly infected B-cells, and we are currently investigating whether this is the case.

In summary, the data presented in this report demonstrate that K1 internalization occurs through a clathrin-mediated endocytic pathway. K1 protein expressed at the surface is constitutively active and recruits PI3K to the membrane. Activation of PI3K results in internalization of K1 via clathrin. Once internalized, K1 continues to transduce signals, inducing the activation of Akt and downstream-signals. Inhibiting either clathrin-dependent internalization or activation of PI3K, prevents efficient endocytosis and signaling by K1.The signaling of K1 at the plasma membrane is regulated by both surface-expression modulation and compartmentalized signaling. This is yet an additional means for KSHV to control cellular signaling pathways.

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CHAPTER FOUR

Functional Analysis of the R1 Gene of Rhesus Monkey Rhadinovirus

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ABSTRACT

Rhesus Monkey Rhadinovirus (RRV) serves as an *in vitro* and an *in vivo* model for Kaposi's Sarcoma-associated Herpesvirus (KSHV/HHV-8). RRV can be grown to high titers on rhesus fibroblasts and the availability of the RRV lytic system facilitates analysis of viral replication and the contribution of individual open-reading frames to viral fitness. At the left end of the RRV genome is a distinct open reading frame designated R1 whose position is equivalent to that of the saimiri transforming protein (STP) of herpesvirus saimiri (HVS) and the K1 protein of KSHV. The cytoplasmic tail of R1 contains motifs capable of binding to the SH2 domains of protein kinases similar to K1. Like K1, R1 has previously shown to be capable of activating B lymphocyte signaling and interacting with the major B cell kinase, Syk. Using a recently available genetic system consisting of a set of overlapping cosmid spanning the entire RRV genome, we deleted the R1 ORF from the RRV genome, replacing the R1 ORF with a Green Fluorescent Protein (GFP) expression cassette. We have analyzed the integrity of the viral genome and performed preliminary analysis of the replication kinetics and the ability of the recombinant virus to establish latency. Creation of this virus gives us a powerful tool to analyze the role of R1 in the RRV life-cycle.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (7, 8, 29). A related simian virus, rhesus monkey rhadinovirus (RRV) was isolated from rhesus macaques at the New England Primate Regional Center, deemed RRV H26-95 (11). Sequence analysis revealed that genomes of KSHV and RRV26-95 are co-linear, with the majority of coding genes in the same genomic locations and in the same orientation. The two viruses are more similar to each other than any other herpesvirus (1). An RRV isolate, RRV 17577, has also been identified by the Oregon Primate Research Center (27).

RRV serves as both an *in vitro* and *in vivo* model for KSHV. Majority of the open reading frames (ORFs) involved in KSHV pathogenesis are also encoded by RRV. Studies of many of the RRV ORFs have shown that they function similarly to their KSHV homologues. *In vivo,* it has been shown that experimental infection of rhesus macaques with RRV results in a lympadenopathy, similar to MCD (24). In the context of a dual infection with simian immunodeficiency virus (SIV), the animals developed lymphoproliferative disorders and B-cell hyperplasias (36). Given the similarities in their ORFs and associated diseases, RRV can serve as a model to study the contribution of individual ORFs to KSHV-related diseases.

The first open reading frames of KSHV and RRV encode proteins called K1 and R1, respectively (10, 20, 23). R1 shows limited sequence homology to K1 in its extracellular domain. The amino-terminal extracellular domains of both K1 and R1 closely resemble those of the members of the immunoglobulin receptor superfamily (10,

23). Similar to K1, R1 has been shown to transform rodent fibroblasts and to functionally replace the Saimiri Transforming Protein C (STP) of Herpesvirus Saimiri (HVS) in immortalizing common marmoset peripheral blood mononuclear cells to IL-2-independent growth *in vitro*. Injection of R1-expressing rodent fibroblasts into nude mice resulted in the formation of multifocal and disseminated tumors in these mice (10). While the extracellular domains of R1 and K1 structurally resemble each other, the cytoplasmic tail of R1 is significantly longer than that of K1 and contains several potential SH2 binding motifs which function as immune-receptor tyrosine-based activation motifs (ITAMs) (9). Like K1, R1 is capable of activating B lymphocyte signal transduction as evidenced by tyrosine phosphorylation of cellular proteins, mobilization of calcium from intracellular stores and activation of the transcription factor NFAT (9).

The ability of RRV to replicate in rhesus fibroblasts allows for the study of the contribution of individual open reading frames to overall viral replication. In order to do this, genetic manipulation of the viral genome is crucial. The conventional means to do this is through homologous recombination in eukaryotic cells, which allows for the introduction of specific mutations in the viral genome. Although this is possible with RRV, it is often laborious. Additionally, it can be difficult to isolate recombinant viruses away from wild-type virus. However, there have been two recently described systems that allow for genetic manipulation of RRV: (i) creation of a bacterial-artificial chromosome (BAC) containing the RRV 17577 genome (17) and (ii) a set of overlapping cosmids incorporating the RRV H26-95 genome (5). Our lab has been involved with making the overlapping cosmid system using RRV H26-95 (5) and we chose this system to make a recombinant RRV that is deleted for R1.

The cosmid system was developed by Bilello et al (5). Overlapping cosmid clones were generated that encompass the entire 130 kb genome of RRV H26-95, including the terminal repeat regions required for viral replication. They showed that the cloned RRV that was produced by co-transfection of overlapping cosmids spanning the entire RRV H26-95 genome replicated with growth kinetics and to titers similar to those of the parental, uncloned, wild-type RRV H26-95. We have utilized this genetic system to generate an R1-null GFP expressing recombinant virus to determine the role of R1 in the viral life cycle.

MATERIAL AND METHODS

Cell culture. Rhesus Fibroblasts (RhF's) were immortalized as previously described (13). RhF's, 293, 293Ts, and 293-RRV-GFP cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified eagle medium H with Gluta-max supplemented with 10% fetal bovine serum. BJAB and BJAB-RRV-GFP cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVECs) immortalized with hTERT, previously described (34), were cultured in sterile endothelial growth medium with 10% FBS. Construction of RRV-GFP_{CC} and RRV-J has been previously described (5).

For stably expressing R1 constructs in RhF's, 3.0 μg DNA was transfected into RhF's using the Amaxa Nucleofection system as described by the manufacturer (program U-23). Following transfection, cells were recovered in complete media for 48 h. Cells were then maintained in complete media plus 1mg/mL G418 for approximately two weeks. Cells were analyzed for recombinant gene expression by western blot.

Establishment of latently and persistently infected cell lines. BJAB, 293 cells or HUVECs, were infected with RRVΔR1-GFP$_{CC}$ or RRV-GFP$_{CC}$ at a multiplicity of infection (MOI) of >1 in the presence of 4 μg of Polybrene/ml (Sigma Chemical Company) for 1 h. The virus inoculum was removed, and cells were resuspended in appropriate complete media. Cells were passaged without selection and monitored for fluorescence.

Plasmids. R1$_{WT}$ and cytoplasmic deletions were obtained by PCR using *BamHI* and *XbaI* containing primers, using pFJ-R1 expression plasmid (previously described(10)), as template. R1 constructs were subcloned into the pCDEF3 vector. All R1 constructs contain a C-terminal AU1 tag.

Cosmid construction. Construction of the RRV H26-95 cosmid library has been previously described (5). Briefly, the cosmid vector, pSCos/ICeuI, used to make libraries was derived from pSuperCos 1 (Stratagene, La Jolla, CA), with a modified aptomer containing ICeuI-BamHI-ICeuI sites. Cosmid libraries were constructed from purified RRV DNA according to the instructions of the SuperCos 1 Cosmid Vector Kit (Stratagene).

To generate RRVΔR1-GFP$_{CC}$, a portion of the R1 ORF was amplified from the ah28ΔA/H cosmid with primers: 5'-CAGCTGGGATCCACTAGTAGTAACACATAGT ATTTC-3' and 5'-CAGCTGGAATTCATTTAAATGATTGTACTCATTGTG-3'**.** Amplified product was cut with BamHI and EcoR1 and ligated into pSP72 vector at same sites (R1-RRVpSP72). Site directed mutagensis was performed to insert a SmaI site in the R1 ORF using primers: 5'-CACCACATCCCGGGGATACCTACTTGC-3' and 5'-GCAAGTAGGTATCCCCGGGATGTGGTG-3' using QuikChange site-directed

mutagenesis kit (Stratagene) as directed by manufacturer. Mutation was verified by DNA sequencing (R1-SpeI-SmaI pSP72). An EGFP expression cassette was inserted into the generated SmaI site by amplifying the CMV promtor, EGFP and polyA region from EGFPN-1 vector (Clontech) using primers 5'-GGATATCGTAATCAATTAC GGGGTCAT-3' and 5'-TGGATATCACCACAACTAGAATGCAGTG-3' (ΔR1/GFP pSP72). ΔR1/GFP-pSP72 was then cut with SpeI and SwaI and ligated into the same sites in cosmid AH28Δa/h to generate ah28ΔA/H- ΔR1-GFP.

Reconstitution of recombinant viruses. Prior to transfection, cosmids were digested overnight with the ICeuI homing endonuclease, removing the RRV H26-95 sequence from the pSuperCos-1 backbone vector. The cosmid DNA was precipitated by adding 3 volumes of 5% 3 M sodium acetate–95% ethanol and incubating for 1 h at -20°C. The DNA was then pelleted by centrifugation for 10 min at maximum speed in a microcentrifuge. The pellets were washed in 70% ethanol, dried, and rehydrated in distilled water. One day post-seeding, 293T cells (4.5×10^5 cells/well in six-well plates) were transfected with a combination of digested overlapping cosmids, ah28ΔA/H- ΔR1-GFP, #15A, #1, #37 and ah34 (Figure 1) (0.4 μg of each cosmid) using Transfectin reagent (Bio-Rad Laboratories, Hercules, CA) following a scaled down procedure. At 5 days post-transfection, media was harvested and centrifuged at 2000 rpm for 10 minutes to remove debris. The media from the three identical cosmid transfections were combined and stored at 4C.

To amplify recombinant stocks generated in 293T cells, RhF's were split and 24 hours later were inoculated with the entire culture supernatant from the 293T transfections. Seven days later, the infected cells were split 1:3 and monitored daily for

the appearance of GFP and/or viral plaques. Cultures were maintained for 2 weeks before clarified culture media was harvested. Fresh RhF's were inoculated with supernatant from the first passage described above, and maintained without splitting until complete lysis of the RhF monolayer.

Revertant virus creation. To generate revertant (marker rescue) virus, cosmid AH28Δa/h was digested o/n with ICeuI and precipitated as above. Approximately 0.5 μg DNA was transfected into RhF's using the Amaxa Nucleofection system as described by the manufacturer (program U-23). Following transfection, cells were recovered in complete media for 24 h. RRVΔR1-GFP_{CC} recombinant virus was used to infect the transfected RhF's at an MOI greater than 1. After complete CPE, clarified supernatants were harvested and replated on fresh RhF monolayers. GFP-negative plaques were picked and replated a minimum of 3 times, then allowed to incubate until complete lysis was observed. 17 different revertant viral supernatants were harvested and viral DNA purified using DNeasy kit (Qiagen).

Analysis of viral integrity: Virus was harvested from cell-free media from virus infected cells by centrifugation at 15,000 rpm for 3 h. Virus pellet was resuspended in PBS and treated with proteinase K for 2 h at 56°C. Viral DNA was extracted by phenol chloroform method.

Viral DNA was subject to PCR analysis with the following primers: R1 flanking primers: 5'-CCGTTGTGGTTACAATACACCTG-3' and 5'-TGAACCACCGCACGG AGC-3', R1 internal primers: 5'-GGGGGTACCCTTCAACCTGTATCGGTGGAGC-3' and 5'-TTGATGATTCAGAGTTCTCGTTGC-3' and GFP internal primers: 5'-CCTGG TCGAGCTGGACGG-3' and 5'-GATCGCGCTTCTCGTTGGG-3'.

For Southern blot analysis, viral DNA was digested with NotI. Digestions were electrophoresed through 1% low-melt agarose (NuSieve) 1xTBE and transferred to a nylon membrane (Hybond N+) by capillary action. Following UV cross-linking, membranes were pre-hybridized in QuikHyb solution (Stratagene) at 65°C for 15 min. Probes were generated from RRV-DHFR and GFP by random prime method using ^{32}P-dCTP (Roche). Probes were denatured at 95°C for 3 min and added to pre-hybridization solution. Hybridization was carried out for 16 h at 65°C. Probe solution was removed and membranes were washed twice for 15 min with 2xSSC 0.1% SDS at room temperature and once for 30 min at 60°C with 0.1xSSC 0.1% SDS. Membranes were exposed and read on a PhosphorImager Scanner (Molecular Dynamics).

Plaque assays. $2x10^5$ RhF's were plated in 12-well plates. 10-fold serial dilutions of virus-infected cell supernatants were made in DMEM-H supplemented with 2% bovine calf serum (BCS). Each sample dilution was performed in triplicate. Two hundred microliters of each dilution were overlayed on each well of 12-well dishes and incubated at 37°C for 1 h with redistribution of inoculum every 15 min. Inoculum was removed and cells were overlayed with 1.5 ml of DMEM plus 1.5% methyl- cellulose (Sigma M0512), and 2% BCS. Plaque assays were incubated 7 days at 37°C and 5% CO_2. Overlay media was aspirated and staining solution (0.8% crystal violet (Sigma C3886), 50% ethanol) was added to each well and incubated 10 min. Plaques were counted using 10x magnification.

Quantitative real-time PCR. At the indicated time post-infection (p.i.), viral DNA was isolated from 200µl of cell-free culture supernatant from each sample using the DNeasy Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Ten-fold serial

dilutions (ranging from 1 to 10^6 plasmid copies/reaction) of pcDNA3-RRVOrf50 were used in each assay to generate a standard curve for genome copy number. SYBR® Green Real-time PCR using the ABI PRISM® 7900 Sequence Detection System (Applied Biosystems Inc: Foster City, CA) was used to quantify viral DNA. Primers for amplification of an 81-bp amplicon internal to the Orf50 sequence were 5'-GTGGAAAGCGGTGTCACAGA-3' and 5'-TGCGGCGGCCAAAAT-3'. Reactions were run in 384-well format with the following conditions: 95°C for 15 min, followed by 95°C for 15 s, 60°C for 1 min repeated for 40 cycles.

Western blot analysis. Cells were harvested and lysed in RIPA buffer containing protease inhibitors. Cells were freeze/thawed 3 times. Protein concentrations were determined by Bradford Assay. 80μg of protein were loaded per lane and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Membranes were probed with monoclonal mouse anti-AU1 (Covance) and anti-mouse HRP (Jackson ImmunoResearch). Immunoblot detection was performed using SuperSignal Pico West chemiluminescent reagent (Pierce Biotechnology).

RESULTS

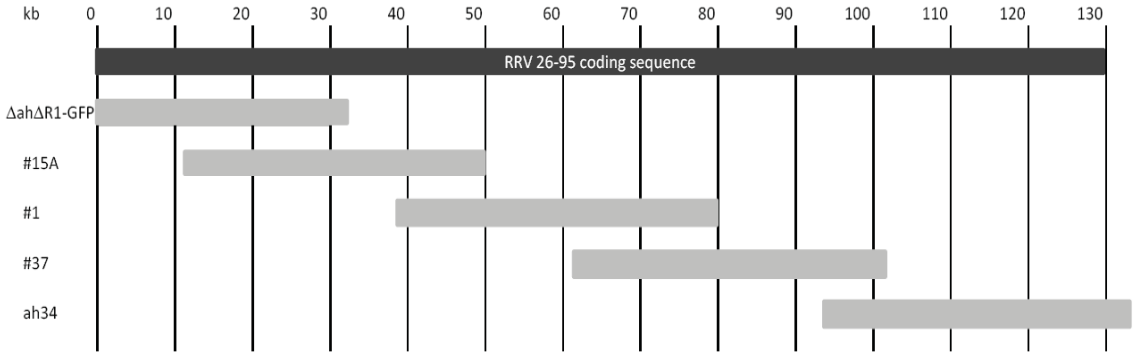
Construction of R1 deletion virus. In order to discern the role of R1 in the RRV lifecycle, we set out to delete the R1 open reading frame (ORF) from the H26-95 genome. We took advantage of the recent advent of a cosmid derived genetic system developed by Bilello *et al* (5). Using cosmid ah28/ΔAH, a GFP expression cassette was inserted into the R1 ORF at a newly generated SmaI site, located approximately 100bp downstream from the R1 start site, interrupting the R1 ORF (Figure 1A). ah28ΔA/H-

ΔR1-GFP was co-transfected with cosmids #15A, 1, 37 and ah34 (Figure 1B) into 293Ts and amplified in RhF's as described above.

Analysis of RRVΔR1-GFP$_{CC}$ genome integrity. Purified viral DNA from RRVΔR1-GFP$_{CC}$ and wild-type H26-95 were subject to PCR analysis to ensure proper placement of the GFP insertion. Primers were designed to amplify regions within the GFP ORF, within the R1 ORF and flanking the R1 ORF. Included as controls are the ah28ΔA/H-ΔR1-GFP cosmid and the ah28ΔA/H parental cosmid. As can be seen in Figure 2, lanes 2 and 6 show amplification of the GFP region showing a band of 517nt, which have as template RRVΔR1-GFP$_{CC}$ and ah28ΔA/H-ΔR1-GFP, respectively. The R1 internal primers have amplified bands of 2322nt for RRVΔR1-GFP$_{CC}$ and ah28ΔA/H-ΔR1-GFP, and 712nt for H26-95 and ah28ΔA/H cosmid (lanes 3 and 7). R1 flanking primers have amplified bands of 3415nt for RRV ΔR1-GFP$_{CC}$ and ah28ΔA/H- ΔR1-GFP and 1786nt for H26-95 and ah28ΔA/H cosmid.

Following PCR analysis, the viral DNA was subjected to restriction digest. 10μg of each viral DNA prep was digested with NotI or HindIII for 3 hours at 37^{o}C. Digested DNA was subjected to electrophoresis through 1% agarose, stained with ethidium bromide. As can be seen in figure 3B, the restriction fragment profile of RRVΔR1-GFP$_{CC}$ DNA with NotI has a band of altered mobility of 6498bp and 2003bp, as compared to wild type H26-95, which is due to the insertion of a NotI site downstream of the GFP ORF (Figure 3A). H26-95 has no such site, rather the band at 7757bp results from the 5'TR to the first NotI site in the genome. The HindIII fragment profile of RRV ΔR1-GFP$_{CC}$ is mirrored by that of H26-95, as they have similar cut sites (Figure 3C).

A



B

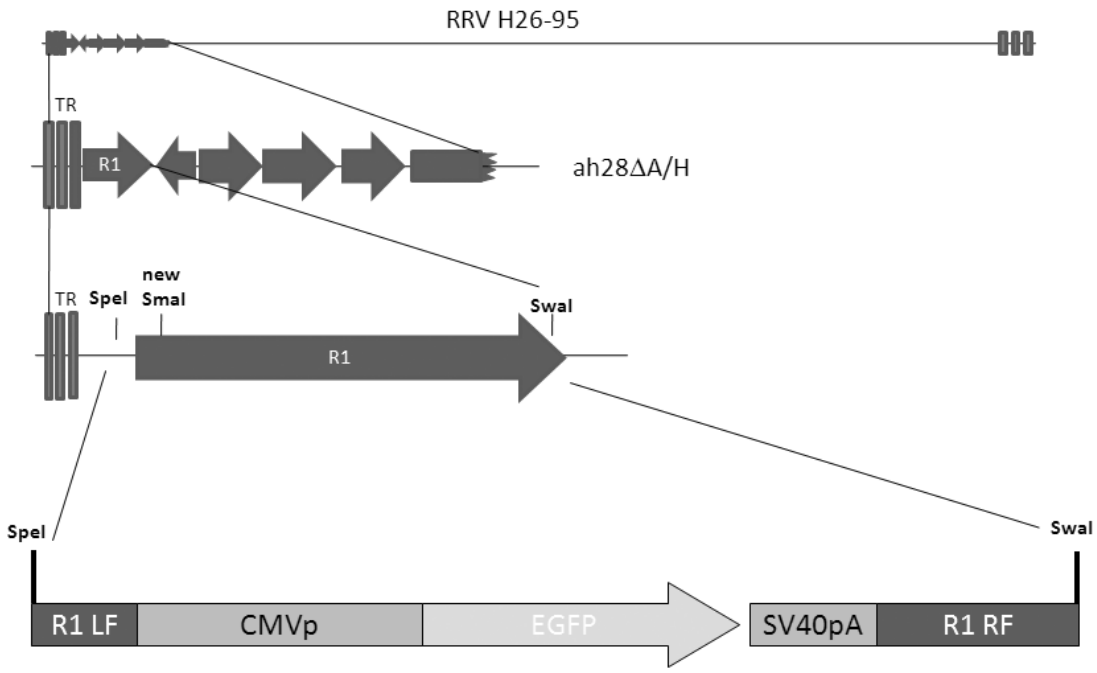


Figure 1. Construction of RRVΔR1-GFP$_{CC}$. A. Insertion of GFP expression cassettes into the RRV ah28ΔA/H cosmid. A SmaI site was introduced into the R1 ORF, within ah28ΔA/H cosmid by site-directed mutagenesis. A cassette containing CMV-GFP and flanked by EcoRV restriction sites was cloned into the SmaI site of ah28ΔA/H to generate ah28ΔA/HΔR1-GFP. The size of the CMV-GFP cassettes is 1619bp. **B.** Schematic representation of the cosmids used to make RRVΔR1-GFP$_{CC}$. The gray bars indicate the extent and location of each cosmid insert within the parental RRV26-95 genome (top black bar). Each cosmid insert was cloned into the pSuperCos 1 vector following the addition of an ICeuI homing endonuclease linker.

To verify that the insertion of exogenous sequences occurred only at the desired location, we performed Southern hybridization analysis of the NotI digested DNA gel. Probe A was generated which was complementary to the RRV DHFR ORF. As expected the DHFR probe hybridized to the 6498bp fragment of RRVΔR1-GFP$_{CC}$ and to the 7757bp fragment of H26-95. Probe B is complimentary to the GFP ORF. Figure 3B depicts the Southern blot performed on the samples using Probe B and shows that the GFP cassette in the RRVΔR1-GFP$_{CC}$ recombinant virus was inserted in the correct site and, as expected, was absent in H26-95 RRV DNA.

Construction of a revertant virus of RRVΔR1-GFP$_{CC}$. A revertant virus was constructed using RRVΔR1-GFP$_{CC}$ and cosmid ah28ΔA/H, as a control for the possible introduction of any mutations that occurred during the construction and isolation of RRVΔR1-GFP$_{CC}$. This revertant virus would be expected to exhibit wild-type growth properties, as long as no other discernable mutations were introduced during recombination of the cosmids. Cosmid ah28ΔA/H was linearized with ICeu-I homing endonuclease overnight and precipitated as described above and transfected into RhF's. 24-hours post-transfection cells were infected with RRVΔR1-GFP$_{CC}$. Virus progeny were screened for isolates that no longer expressed GFP. PCR analysis of two revertants were identical to that of H26-95 (Figure 2). One isolate, designated rRRV-2.3.1, was further analyzed by restriction digest and Southern Blot. As expected, the revertant exhibited the same hybridization profile as wild-type H26-95 DNA (Fig. 3B and C).

Characterization of recombinant RRVs in vitro. We next determined if the replication kinetics differed between the recombinant RRVs generated by co-transfection of overlapping cosmids, including RRVΔR1-GFP$_{CC}$, rescue virus rRRV-2.3.1, RRV-

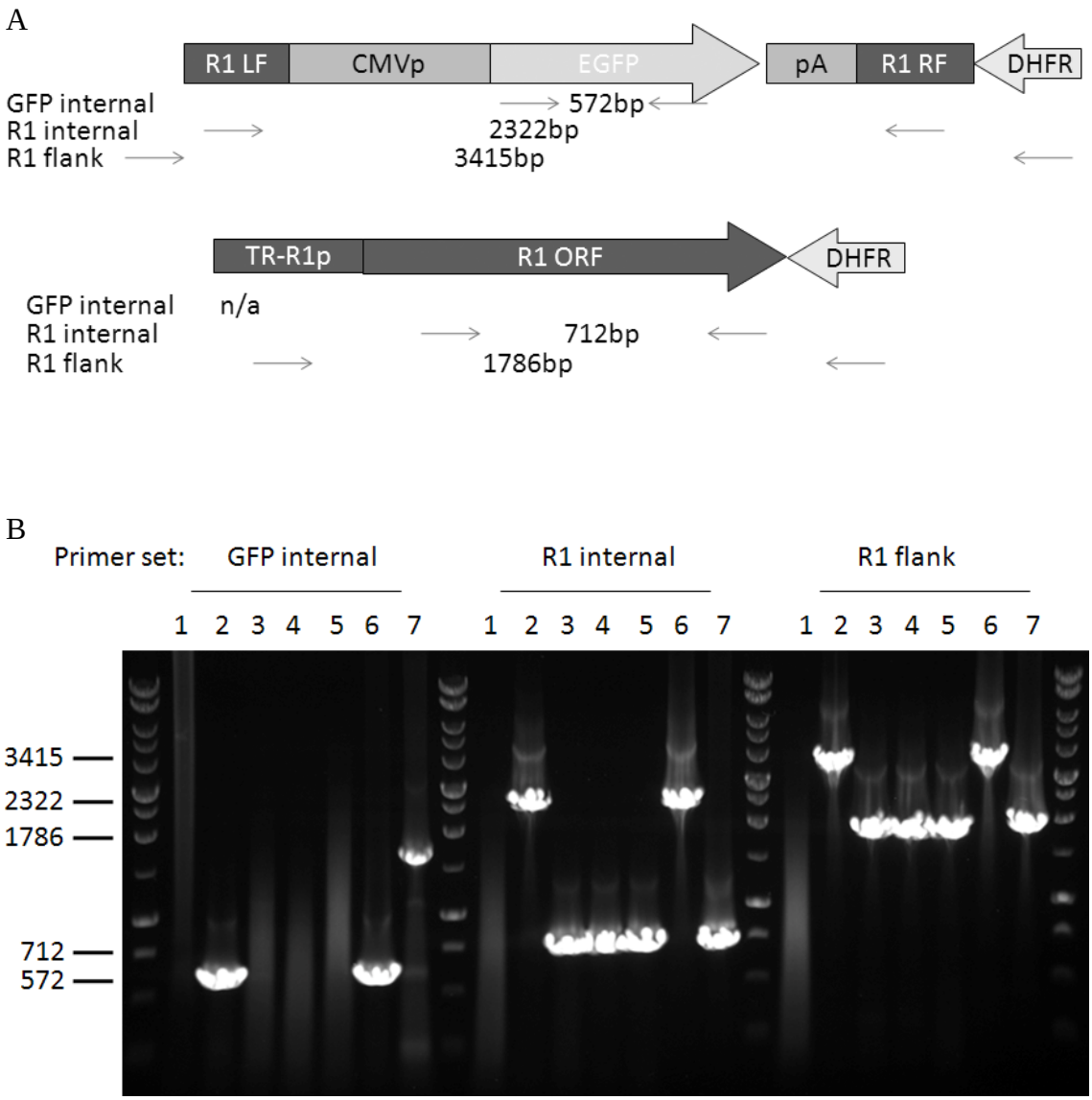


Figure 2. PCR analysis of recombinant viruses. A. Schematic representation of the location of priming sites for templates containing GFP expression cassette (top) and those that do not (bottom). **B.** PCR-amplification was performed using oligos that prime within the GFP cassette (left), within the R1 ORF (middle) or flanking the R1 ORF (right). Templates used are as follows: Lane1: NTC lane 2: RRVΔR1GFPcc DNA lane 3: H26-95 DNA lane 4: rRRV 2.3.1 DNA lane 5: rRRV DNA 4.1.1 lane 6: ΔR1 ah28ΔA/H cosmid lane 7: ah28ΔA/h cosmid.

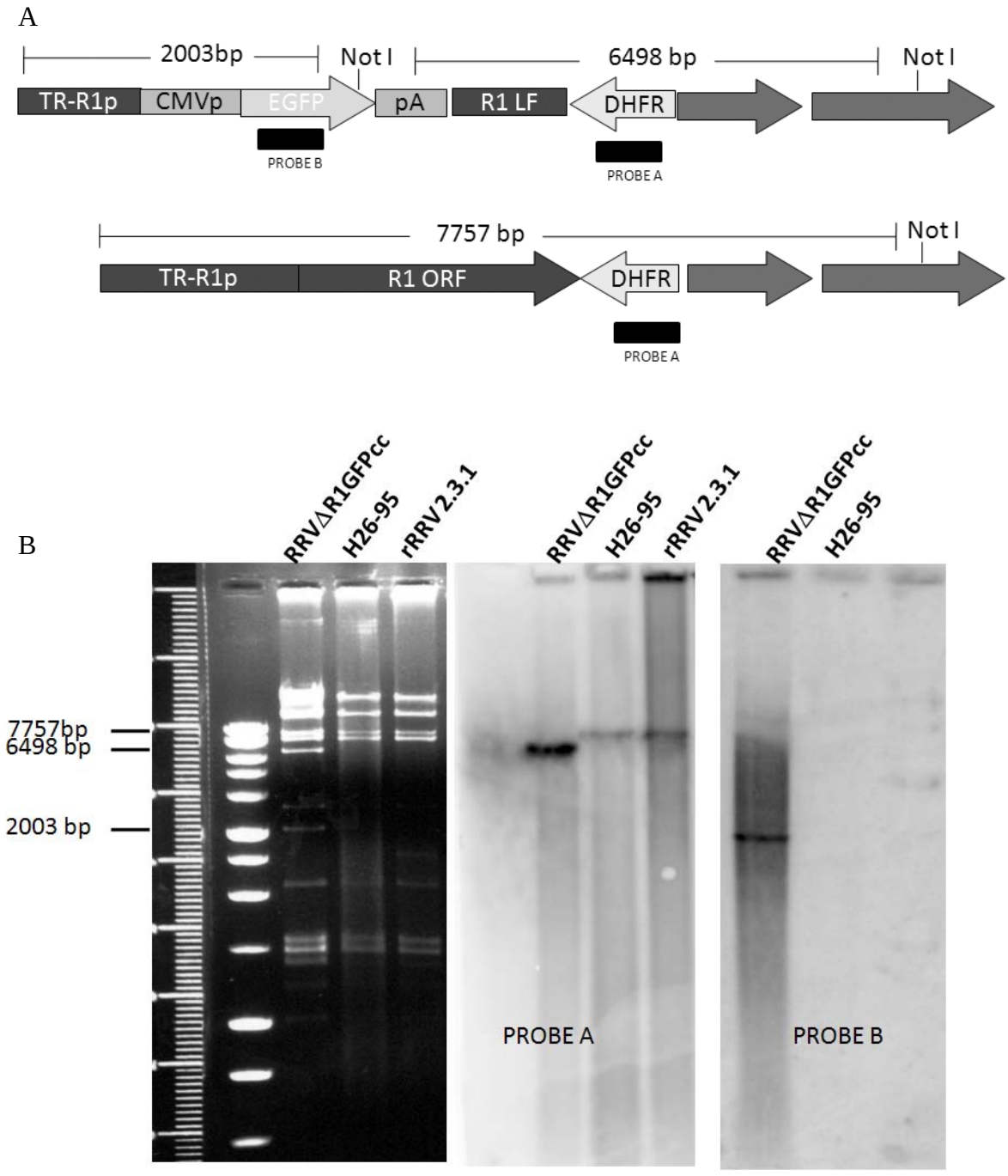


Figure 3. Restriction digest and Southern blot analysis of recombinant viruses. A. Diagram of the 5' end of RRVΔR1-GFP$_{CC}$ (top) and H26-95 genomes, depicting NotI restriction sites and location of probe hybridization sites. **B.** NotI digest (left) and Southern blot of RRVΔR1-GFP$_{CC}$ (lane 1), H26-95 (lane 2) and rescue RRV 2.3.1 (lane 3) genomes probed with RRV DHFR (middle) and GFP (right).

C

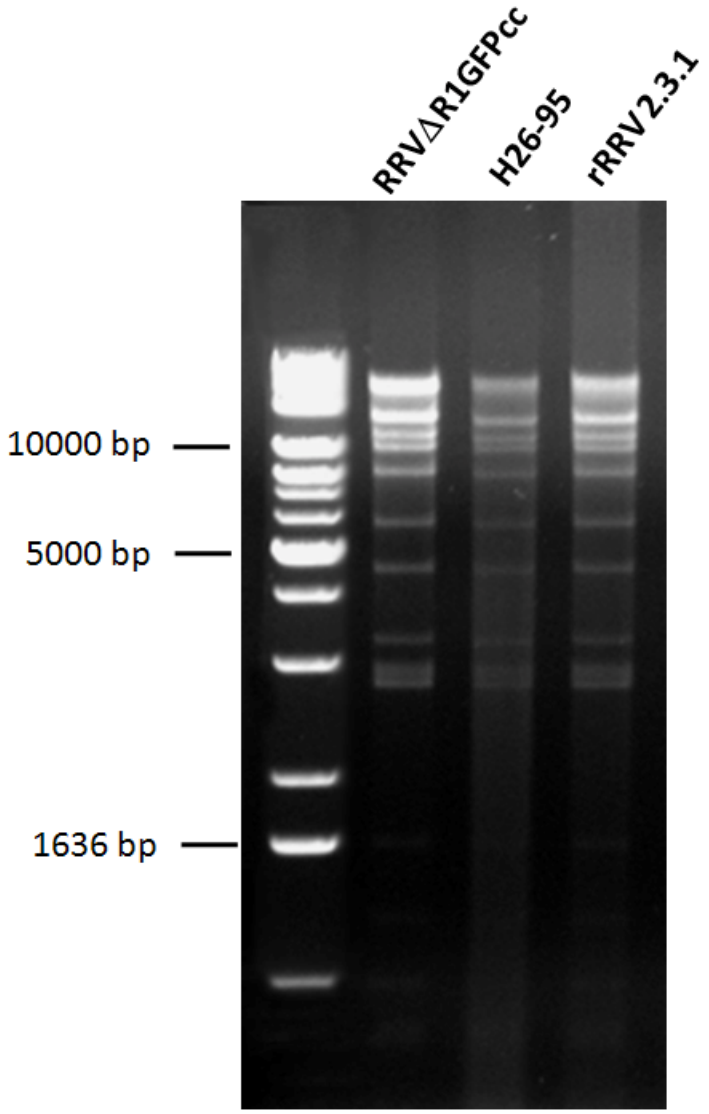


Figure 3 cont'd. C. Restriction digest analysis of recombinant viruses. Hind III digest of RRVΔR1-GFP$_{CC}$ (lane 1), H26-95 (lane 2) and rescue RRV 2.3.1 (lane 3).

GFP_{CC}, a wild-type cosmid derived H26-95 virus expressing GFP, and RRV-J, a wild-type H26-95 (with no trans-genes) made via the cosmid system (5). One day post-seeding, triplicate cultures of RhFs were infected at 0.1 PFU/ cell with RRVΔR1-GFP_{CC}, RRV-J, RRV-GFP_{CC} or rRRV-2.3.1. At each time point, viral supernatants were collected and stored at -80^{o}C. Viral DNA was isolated from each sample and analyzed for RRV genome copies by real-time PCR. Measurement of viral genome copy number by this method was previously shown to correlate strongly with infectious titer using wild-type RRV that did not have gene deletions (14). Preliminary data suggests that the growth rate at each time point was not significantly different between RRVΔR1-GFP_{CC} and RRV-GFP_{CC} (Figure 4) in terms of viral genomes. However, plaque assays may show a difference in viral infectious units. rRRV-2.3.1 shows slightly increased growth rate, especially when compared to the other wild-type virus, RRV-J. This experiment has only been done once at this MOI. Future experiments planned include, repeating this identical experiment, as well as performing this experiment at other MOI's, performing plaque assays with viral supernatants and analyzing the integrity of the RRV-J genome. There is a slight difference in the kinetics of RRV-J and rRRV-2.3.1. Theoretically, these viruses should replicate with similar kinetics. Therefore, confirmation of RRV-J genome by this laboratory is crucial.

Establishment of R1-expressing stable cell-lines. In order to determine the contribution of R1 to RRV lytic replication, we have established a panel of RhF cell lines expressing different deletion mutants of R1 (Figure 5). The many tyrosine residues in the R1 cytoplasmic tail have been analyzed (9), and appear to encode several SH2 binding

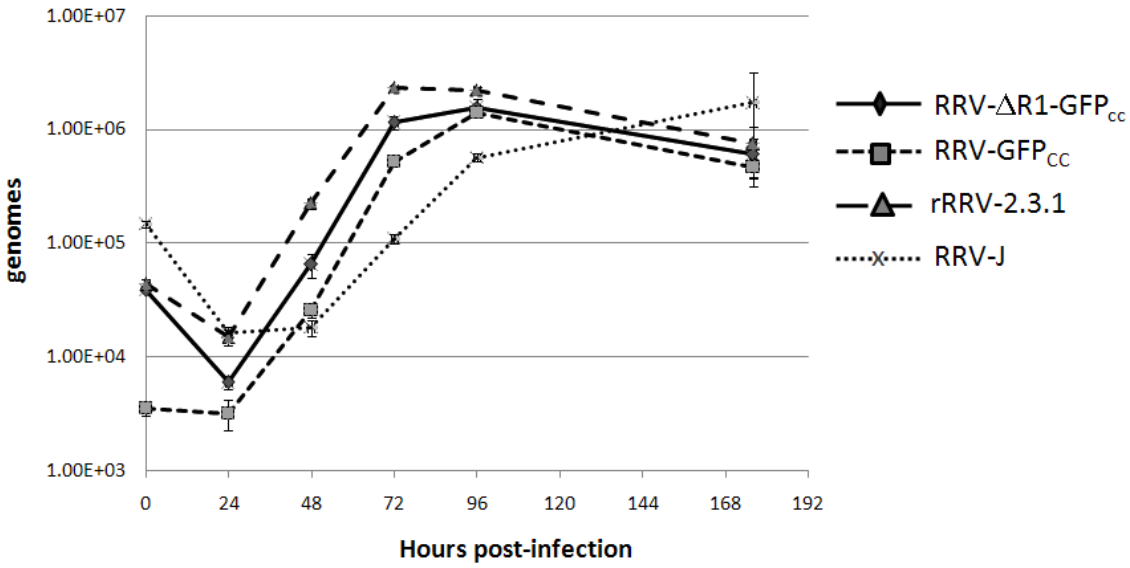


Figure 4. RRV one-step growth curves. Equivalent numbers of rhesus fibroblast cells were infected with RRVΔR1-GFP$_{CC}$ (black diamonds), RRV-GFP$_{CC}$ (grey squares), rRRV 2.3.1(grey triangles) or RRV-J (grey x's) at an MOI of 0.1 and cell-free supernatants were harvested at indicated points post-infection and quantitated by real-time PCR.

motifs. It has been shown that five of the thirteen tyrosine residues of R1 are present within YXXL sequences. YXXL sequence elements have been shown to bind the major B-cell kinase, Syk (19, 21, 28). The third and fourth and the fourth and fifth YXXL motifs resemble ITAMs in that they and the surrounding sequences are spaced in a fashion consistent with that of the ITAM consensus sequence, $(D/E)X_7(D/E)X_2YX_2LX_{7-10}YX_2L/I$. The cytoplasmic deletion mutations were selected based on the types of SH2 binding motifs present in the R1 cytoplasmic domain, as previously described (9). Deletion mutants were cloned from the pFJ-R1 expression (10) and inserted into the pCDEF3 plasmid. Each construct also contains a 3' AU1 epitope. Stable cells were developed by transfecting each construct into RhF and selecting for 2 weeks in G418 containing media. In order to ensure expression of each construct, cellular lysates were subject to western blot analysis. Membranes were probed with anti-AU1 antibody. Shown in Figure 5 is a representative blot. Constucts R1, R1-D3 and R1-D4 were readily detectable. R1-D1 and R1-D2 were detected using a darker exposure (arrows).

These cell lines will allow us to study how the different domains in the C-terminus of R1 may affect lytic replication. By infecting these cells with RRVΔR1-GFP_{CC} and wild-type viruses, we can analyze whether exogenous expression of wild-type and mutant R1 proteins affect viral replication.

Latent cell-lines. In addition to studying the role of R1 in lytic infection, we would also like to analyze its role in latent infection and reactivation from latency. To that end, we have generated several cell-lines in which the virus is latently infected. Our lab has previously shown that RRV latently infects 293 and BJAB cells (12). We have recently generated 293 and BJAB cells infected with RRVΔR1-GFP_{CC} and RRV-GFP_{CC} (Figure

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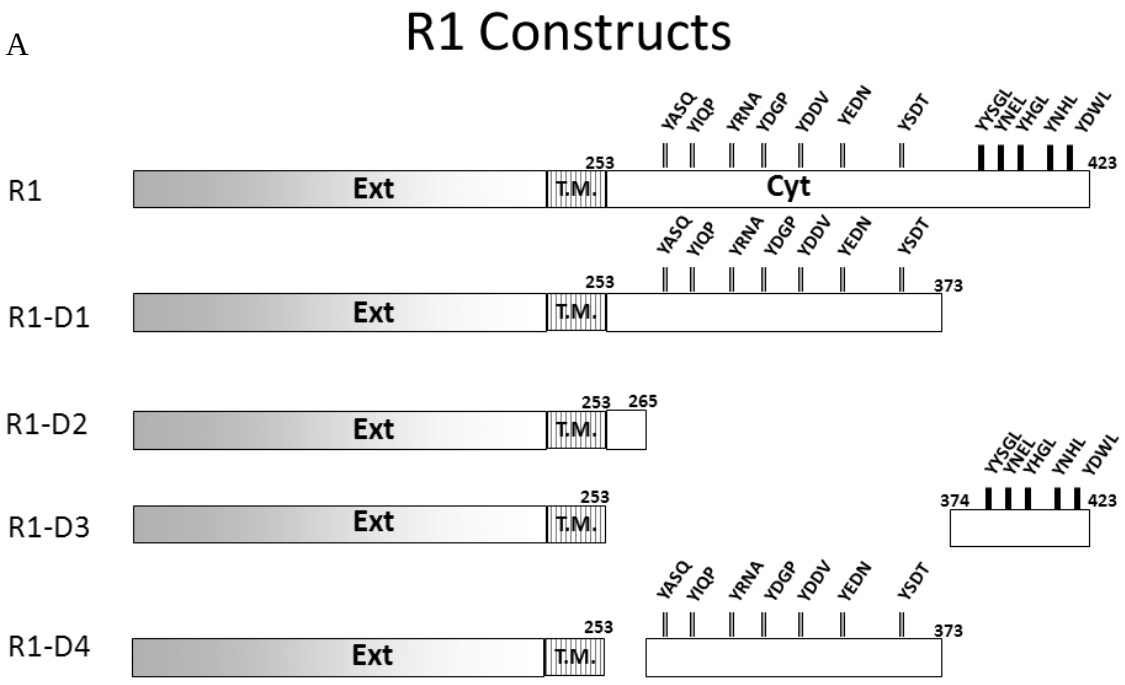

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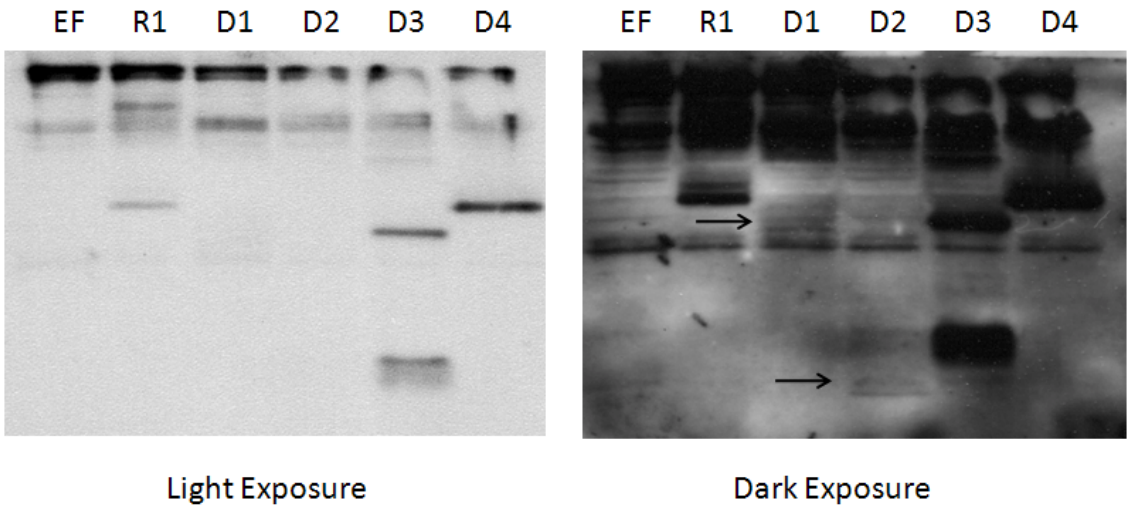


Figure 5. R1 expressing stable RhF cells. A. Panel of R1 expression plasmids. Schematic diagram of wild-type and R1 c-terminal deletion proteins. The shaded box represents the extracellular domain; the open box labeled *T.M.* indicates the transmembrane domain; and the open box represents the cytoplasmic tail. YXXX, tyrosine residues and their surrounding sequences. **B.** Western blot analysis of R1 expressing RhFs. R1 constructs were stably transfected into RhF cells and selected in the presence of 1mg/mL G418. Expression of R1 constructs was analyzed by Western blot. Membranes were probed with anti-AU1 antibody and detected with anti-mouse HRP antibody.

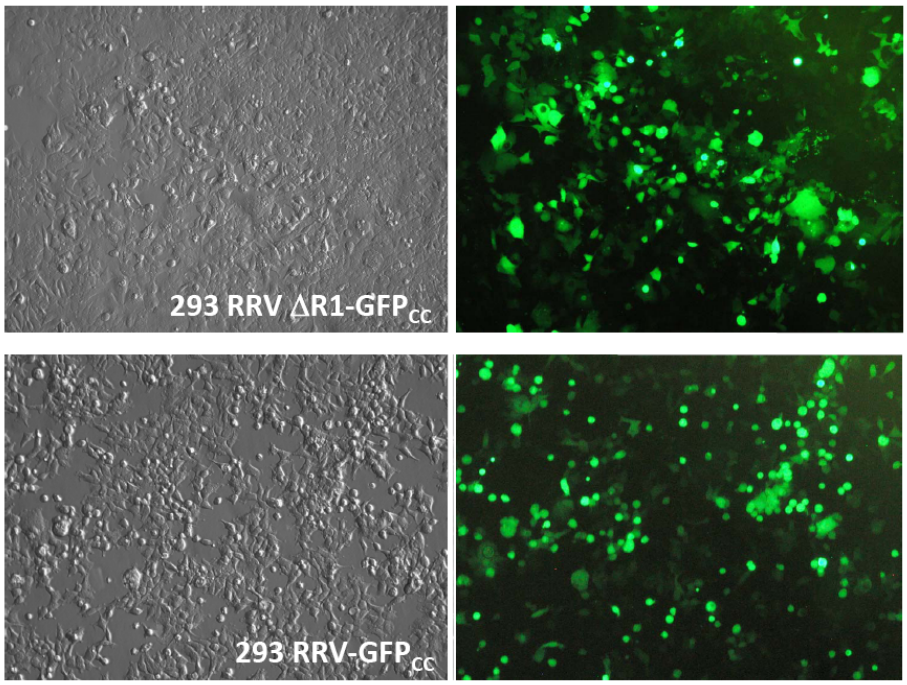
6A and B). By monitoring GFP expression over time, we can analyze the ability of the viruses to establish a latent infection. These cells can also be used to evaluate the ability of the viruses to undergo reactivation.

In addition to the latent cell lines, we have created an endothelial infected cell line that appears to be undergoing a persistent infection by both RRVΔR1-GFP_{CC} and RRV-GFP_{CC}. We have found that HUVECs appear to be persistently infected, that is, lytic replication followed by a period of latency (Figure 6C). To further elucidate if this is true it is necessary in the future to culture the infected cells for a longer period of time. At various time points (normally when the cells are nearly confluent and ready to split), the culture supernatants will be assessed for infectious virus and intracellular-RRV-RNA levels. In addition, we will analyze the ability of these endothelial cells to form tubules using a matrigel-tubule assay to model angiogenesis *in vitro* (6).

DISCUSSION

RRV serves as a model for KSHV, both *in vivo* and *in vitro* (4, 24, 36). KSHV cannot establish a robust and persistent infection in mice or macques (15, 26) and hence, RRV serves as a good model to study KSHV pathogenesis in the host. With the recent advent of genetic systems for RRV, it is now easier to study the contribution of individual ORFs in the development of RRV-associated disease and also provide important insight into the function of homologous ORFs in KSHV and their potential roles in diseases associated with KSHV infection in humans. In this manuscript we have described the construction of a recombinant RRV deleted for the R1 ORF. We have taken advantage of an overlapping set of cosmids developed by Bilello et al (5). When developing the cosmid set they found that the RRV cosmids were too large (>46 kbp including terminal

A



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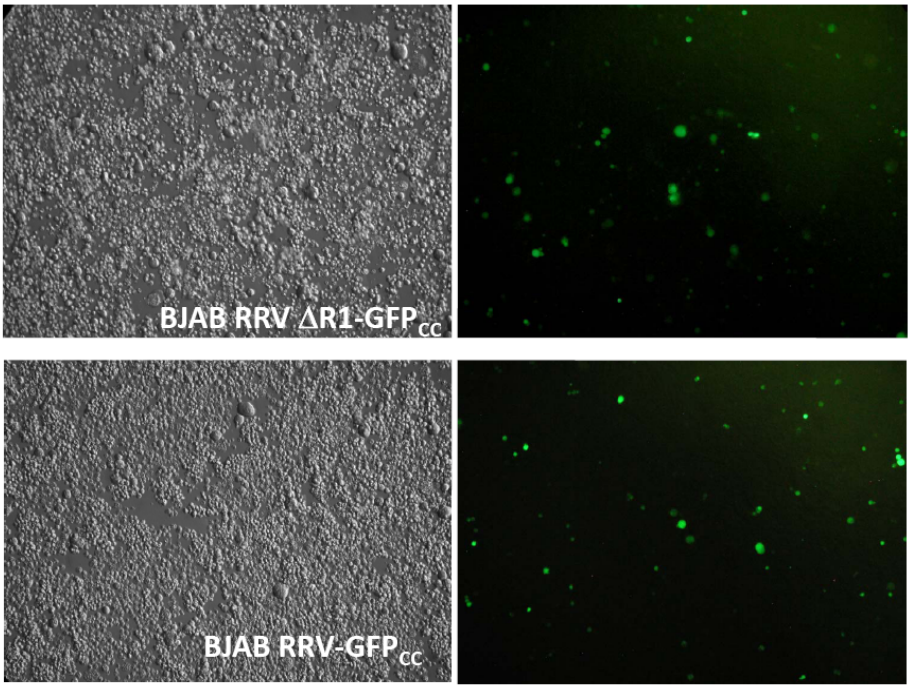

Figure 6. Latently infected cell lines. A. 293 cells infected with RRVΔR1-GFP$_{CC}$ (top) and RRV-GFP$_{CC}$ (bottom). Image taken 14 days post-infection. **B.** BJAB cells infected with RRVΔR1-GFP$_{CC}$ (top) and RRV-GFP$_{CC}$ (bottom). Image taken 15 days post-infection.

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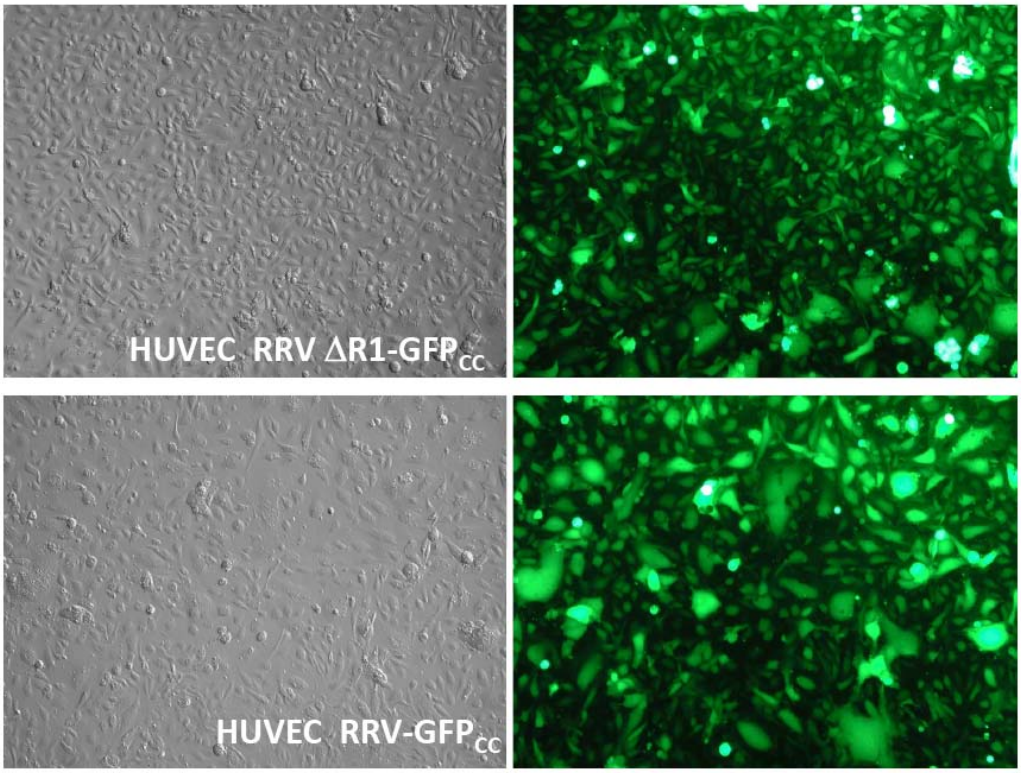

Figure 6 cont'd. Latently infected cell lines. C. HUVECs infected with RRVΔR1-GFP$_{CC}$ (top) and RRV-GFP$_{CC}$ (bottom). Image taken 15 days post-infection.

repeat regions) for routine genetic manipulation. To that end, they generated a sub-cosmid clone by truncation of the existing ah28 cosmid, allowing for insertion of a reporter gene upstream of the R1 promoter. We have utilized this sub-cosmid clone, ah28ΔA/H, for manipulation of the R1 ORF. We generated a unique SmaI site in the R1 ORF in which we subcloned a GFP expression cassette. Via PCR, restriction digest and Southern blot analysis we have confirmed the placement and integrity of RRVΔR1-GFP_{CC}. This recombinant appears to be identical to the wild type RRVH26-95 genome, except for the insertion of the GFP expression cassette. We have also generated a rescue virus, in which the GFP expression cassette was removed via traditional homologous recombination between the RRVΔR1-GFP_{CC} and ah28ΔA/H cosmid.

Characterization of the resultant RRVΔR1-GFP_{CC} and revertant viruses in rhesus fibroblasts demonstrated that these recombinant viruses were replication competent, and replicated in rhesus fibroblasts with growth kinetics as measured by viral genome titers that were similar to those of wild-type viruses. Future studies will determine if this is true at multiple MOIs, as determined by both plaque assay and real time PCR. We will also determine how a panel of R1 mutants may complement the viral replicative capacity of RRVΔR1-GFP_{CC} and other recombinant viruses on stable R1 expressing RhFs.

In the context of KSHV it was found that most adherent cell lines (TIME, SLK, HFF, 3T3, CV-1, 293, and HeLa cells), irrespective of species of origin or tissue lineage, are permissive for viral entry and the establishment of latency (3). Other than RhF cells, this has proven true for RRV, as well. We have found that RRV efficiently infects 293s BJABs and HUVECs. Following KSHV infection, DMVEC (Dermal endothelial cells immortalized with the E6 and E7 genes of HPV type 16) shape changed from a classical

cobblestone to a spindle shaped morphology (25). We have not observed this phenomenon in our RRV infected HUVEC cells. An *et al* found that for telomerase-immortalized human umbilical vein endothelial (TIVE) cells, the majority of cells lose the KSHV DNA over time. However, a small subset of KSHV infected cells were able to maintain the viral episome for >10 months (2). These long-term cells were able to form tumors in nude mice. It will prove interesting to determine if our RRV-GFPcc HUVEC cells also form tumors in nude mice and how they may differ from our RRVΔR1-GFP_{CC} HUVEC cells. Perhaps R1 will prove to be important for the development and maintenance of tumors over time in a xenograft model.

Unlike KSHV, we have found that RRV efficiently infects lymphocyte cells lines, both BJABs and DG-75s (data not shown). This can serve as an attractive model to study RRV in a more relevant, latently infected cell line. Mansfield et al found that *in vivo*, RRV H26-95 preferentially infected $CD20^+$ B lymphocytes (24). Studies analyzing latency and reactivation will be important and may help determine what role R1 may play in the viral lifecycle.

It is possible that R1 does not play a role in lytic replication, establishment of latency or reactivation in *in vitro* models. Instead, R1 may function more as an immunoregulatory protein and will be important in an *in vivo* model. It has been shown that K1 of KSHV functions to down-regulate the surface expression of the B-cell receptor (BCR) (22). Activation of the BCR initiates multiple intracellular signals that often lead to apoptosis. Inhibition of BCR-mediated signaling by the down-regulation of its surface expression or modulation of BCR signal transduction may provide a long-term survival advantage *in vivo*. Additionally, we and others have shown that K1 can function as an

anti-apoptotic protein, protecting cells from Fas-mediated apoptosis (31, 35). Published data has shown that R1 activates B-cell signaling pathways similarly to K1 (9). Whether R1 affects BCR expression has not yet been determined. However, we have seen that R1 activates the Akt pathway, (Figure 7), similarly to K1 (31, 33, 35), which can control Fas-mediated apoptosis (30, 32); further suggesting that R1 has a similar phenotype to K1 *in vitro.*

It will prove interesting to analyze experimental infection of rhesus macaques with RRVΔR1-GFP$_{CC}$ as compared to wild-type viruses, with and with-out co-infection with SIV. It has been shown in the context of SIV co-infection, that experimental infection with wild-type RRV, results in B cell hyperplasia, persistent lymphadenopathy, and persistent infection in macaques (18, 24, 36). Studies involving a related gamma-herpesvirus, HVS, deleted for the left-hand terminal membrane protein, STP or Tip, resulted in replication-competent deletion mutant that were shown to not be required for viral replication or persistence but were essential for growth transformation of primary T cells in culture and for disease induction *in vivo* (16). RRVΔR1-GFP$_{CC}$ may prove to have an interesting phenotype *in vivo,* perhaps in the context of the development of B-cell hyperplasia, since R1 deregulates signal transduction in B-cells (9). Thus, R1 may be important for the development of RRV-associated disease *in vivo.*

In summary, we have developed a recombinant RRV, in which the R1 ORF has been interrupted with a GFP expression cassette. Determining the contribution of individual ORFs using an RRV genetic system is a logical way to surmise the role of homologous ORFs of KSHV to viral pathogenesis. This virus, RRVΔR1-GFP$_{CC}$, will be used as a model to study the role of R1 in the viral life-cycle, both *in vitro* and *in vivo.*

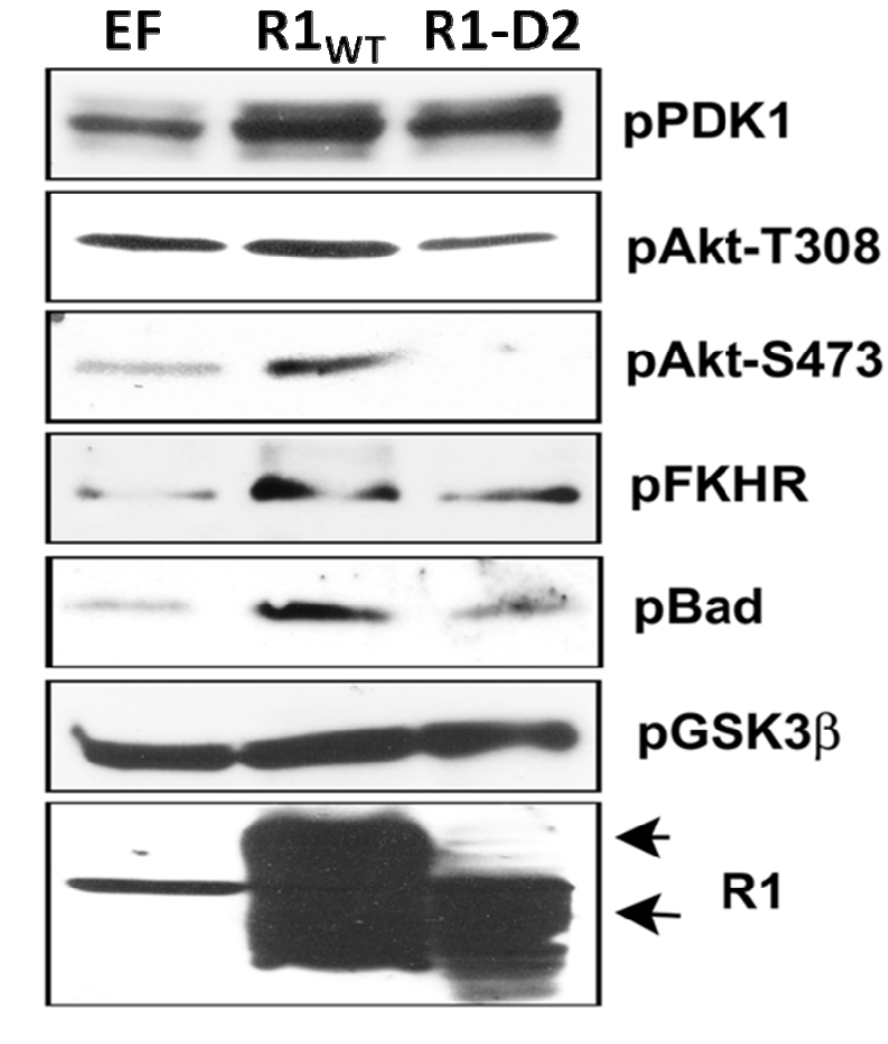

Figure 7. R1 activates the Akt pathway. 293 cells were transiently transfected with empty vector (EF), $R1_{WT}$, or R1-D2, which is a c-terminal deletion mutant. Cells were serum starved for 48hours, lysed and run on SDS-PAGE (each lane loaded with 80ug protein). Membranes were probed with the following antibodies: phospho-PDK1, phospho-Akt Thr308, phospho-Akt Ser473, phospho-FKHR Ser256, phospho-Bad ,phosphor-GSK3β (all from Cell Signal Technologies), and anti-Au1–HRP (Bethyl Laboratories).

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CHAPTER FIVE

GENERAL CONCLUSIONS

General Conclusions

The gene products of oncogenic DNA viruses can alter multiple cellular pathways and operate at various points in the signaling cascades promoting cell growth and proliferation. Since its discovery 13 years ago, much effort has been devoted to the study of KSHV. KSHV is now consistently detected in all forms of Kaposi's sarcoma (KS), primary effusion lymphomas, and multicentric Castleman's disease (1). KSHV encodes numerous proteins that deregulate cellular signaling pathways, to aid in viral infection, replication and life-long persistence in the host. Expression of these proteins contributes to the onset of KSHV-associated diseases.

Once such protein, K1 is the subject of this dissertation. Encoded by the first ORF of KSHV, K1 is one of the most variable proteins in the KSHV genome. K1 has been shown to deregulate lymphocyte signaling, transform cells and down-modulate BCR expression (14, 16, 24). K1 expression has been found in all KSHV-related neoplasms, although the percentage levels of K1 gene expression varies by tumor type. K1 has been shown to be highly expressed during KSHV lytic replication (4, 13). In latent cells, K1 RNA is expressed at lower levels (4). Interestingly, Bowser *et al* (4) have shown that the native K1 promoter is active in many different cell lines in the absence of the KSHV Orf50/RTA protein and that RTA can activate the K1 promoter through three RTA responsive elements in the K1 promoter. This raises the possibility that in latent cells, K1 expression may be regulated by environmental stimuli such as cytokines or growth factors.

We and others (16, 37) have shown that K1 increases the phosphorylation and activation of the p85 subunit of PI3K. The work presented in chapter 2 details our

investigation into the consequences of this interaction; mainly what affects K1 has on down-stream signaling by PI3K, particularly in B-lymphocytes. K1 expression in BJAB B-cells was found to induce the activation of Akt, indicated by phosphorylation of Akt on two residues, Thr308 and Ser473. The ITAM of K1 was found to be required for this activation, as mutation of the tyrosines in the ITAM abrogated phosphorylation of Akt. Akt functions to phosphorylate many down-stream targets involved in both cell survival and cell cycle regulation. When cellular targets of Akt in K1-expressing cells were examined, only one target, Forkhead transcription factor (FKHR), was found to be affected, evidenced by an increase in phosphorylation. This phosphorylation is indicative of FKHR's inactivation by Akt (3, 22, 36). The changes seen in the Akt pathway in K1 expressing cells were found to be prevented when cells were treated with LY294002, a PI3K inhibitor (18). This suggests that K1 activates Akt through PI3K.

FKHR, also known as FOXO, is in the winged-helix family of transcription factors. In the absence of cellular stimulation, FOXOs are localized in the nucleus, where they activate transcription of target genes. However, upon activation of Akt by growth or survival factors, FOXO proteins are phosphorylated by Akt at specific sites, eliciting their re-localization from the nucleus to the cytoplasm. Phosphorylated FOXOs bind to 14-3-3 proteins in the nucleus immediately before re-localizing to the cytoplasm, where they remain sequestered to 14-3-3 proteins (3, 5, 12). A number of pro-apoptotic proteins such as Fas ligand (FasL), Bim, TRAIL, and the insulin-like growth factor-binding protein (IGFBP)-1, are transcriptionally regulated by members of the FOXO subfamily (5, 8, 11, 36). FOXO factors also regulate G1 cell-cycle progression by modulating expression of the cyclin dependent kinase inhibitor $p27^{Kip1}$ and D-type cyclins (19, 22, 26, 27).

Cytoplasmic sequestration of FOXOs prevents transcriptional activity, resulting in cell-cycle progression and inhibition of apoptosis.

The apparent inhibition of FKHR in K1-expressing cells led us to analyze FKHR's sub-cellular localization. In both epithelial and B-cells, expression of K1 led to an increase in cytoplasmic localization of FKHR as shown by both immuno-fluorescence and cellular fractionation assays. This led us to analyze the transcriptional activity of FKHR. Utilizing two different FKHR promoter recognition elements, it was shown that K1 expression leads to a repression of luciferase activity driven by FKHR promoter elements.

Because FOXO's are intimately involved in apoptosis, we next sought to analyze how K1 expression influences apoptosis. Over-expression of FKHR results in nuclear accumulation and apoptosis. When apoptosis was induced by over-expression of FKHR, K1 expression resulted in only a limited protection, as measured by Caspase-3 activity. However, when apoptosis was induced by engagement of the Fas Receptor, K1 expression resulted in ~50% protection from apoptosis. As one of the FKHR responsive promoter elements was from the FasL promoter, this was not surprising. Typically, FKHRL1 (FOXO3a) mediates the transcription of FasL in response to apoptotic stimuli. This activation is mediated through binding of FKHRL1 to the three Forkhead responsive elements (FHRE) in the FasL promoter. Secreted FasL binds to the Fas receptor on the surface of the cell and establishes a positive feedback loop resulting in cell death (Figure 1). Interestingly, after the data in Chapter 2 was published, another lab found that K1 expression protects cells from Fas-mediated apoptosis, suppresses formation of the death-

inducing signaling complex (DISC) and blocks caspase 8 activity, but did not suppress apoptosis induced by TRAIL or irradiation (40).

We also found that K1 protected PEL cells latently infected with KSHV from Fas-mediated apoptosis. In PEL cell culture lines, K1 expression is found in a small percentage of cells. By over-expressing K1 in PEL cells, there is an increase in protection of apoptosis, suggesting that K1 can modulate cell-signals to protect virus infected cells from apoptosis.

It is interesting that of the Akt targets analyzed, only FKHR was modified by K1. We used the BJAB cell line, which is a highly transformed Burkitt's lymphoma cell line (20). It is possible that the other targets analyzed have reached a saturation point of activation/inhibition by Akt, before the introduction of K1. Indeed, when K1 expression was investigated in primary HUVECs, all Akt targets analyzed had increased phosphorylation (39). This suggests that if K1 expression was analyzed in primary B-cells, or at least, a less transformed B-cell line, other Akt targets might also be modified.

K1 is not the only ORF of KSHV to target the PI3K pathway. The viral G-protein coupled receptor (vGPCR) also modulates this pathway (21, 30), inducing Akt activation, both *in vitro* and *in vivo.* Other groups have shown the importance of the PI3K/Akt pathway in the context of KSHV-related diseases. This pathway is constitutively activated in several PEL cell lines. Uddin *et al* demonstrated that inhibition of PI3K by LY294002 resulted in decreased phosphorylation of basal levels of Akt, GSK3, and FKHR and induced apoptosis in most PEL cell (38). Additionally, one of the targets of Akt, mTOR, has been indicated as a potential target for KSHV-related diseases (29). It has also been revealed that PI3K plays a role in entry of KSHV into target cells (23, 25).

All of these studies indicate the significance of PI3K signaling to virus survival. Manipulation of the PI3K pathway gives the virus extensive control over many cellular functions. There are a multitude of molecules in the PI3K pathway and discerning the targets of KSHV modulation will lead to a better understanding of viral pathogenesis.

In chapter 3, we analyzed control of cell-surface expression of K1. As there was no information on how K1 surface expression is controlled, we first determined that K1 is endocytosed in a clathrin-dependent manner. We found that the ITAM is required for internalization, as both a $K1_{ITAM-}$ mutant and a C-terminal deletion mutant, are both internalized at a slower rate as determined by ELISA. The ELISA measures internalization after pre-labeling the cell surface with antibody. There is a possibility that the antibody cross-linking of K1 induces endocytosis and hence we also performed an endocytosis assay that was not based on antibody binding to K1. Interestingly, when internalization was measured without antibody pre-labeling, but rather labeling cell surface molecules with biotin, we found that neither the ITAM- mutant nor the C-terminal deletion mutant were internalized at all, further supporting our hypothesis that the ITAM is critical for internalization. K1 internalization analyzed by confocal microscopy gave similar results.

The exact residues involved in internalization of K1 are currently being pursued. For example, the first SH2 binding domain of the ITAM also encodes for a predicted tyrosine-based endocytic motif (YXXL). K1 also has several conserved lysines in its C-terminus. Ubiquitin conjugation to lysine residues is a common modification that signals receptor internalization and degradation. K1 has been shown to induce the phosphorylation of c-Cbl, an E3-ubiquitin ligase. Cbl proteins facilitate the ubiquitination

of activated tyrosine kinases and other signaling proteins. A monoubiquitin tag promotes sorting of activated receptors and associated proteins into internal vesicles of the multivesicular body, facilitating their lysosomal degradation, whereas a polyubiquitin tag promotes proteasomal degradation (reviewed in (35)). Whether or not K1 undergoes ubiquitin modification, either mono- or poly- is currently being investigated. The role of Cbl in K1 signaling and internalization also remains to be revealed.

K1 appears to be internalized in a conventional clathrin-mediated route. Once internalized, K1 traffics to the early endosome. From the early endosome, $K1_{WT}$ appears to traffic to the recycling-endosome, whereas $K1_{ITAM-}$ and $K1_{\Delta C}$ do not. This is interesting as it has been shown that other viral glycoproteins such as Us28 of CMV, (10), ORF74 of KSHV (2) are constitutively internalized and recycled. Constitutive recycling may provide a convenient method to regulate surface receptor numbers. If a rapid decrease in surface receptors is needed, the internalization rate could be increased and/or recycling decreased and vice versa. Recycling promotes immediate cellular internalization without the need for recruitment of the necessary internalization machinery. The receptors can then be more quickly transported to the appropriate intracellular location where signaling occurs. One speculation is that viral proteins need to be regulated to prevent chronic hyper-stimulation of signaling pathways that are not beneficial for viral replication and infection. Mammalian cells have evolved numerous mechanisms to prevent aberrant chronic activation of signaling pathways. By engaging cell regulatory machinery, K1 may be able to signal only to moderate levels, thus potentially avoiding recognition by cell defense machinery. By blocking down-stream signaling of K1 with chemical inhibitors, the internalization rate of K1 was impeded, suggesting the formation of a feed-back loop between signaling and internalization.

It is also possible that internalization functions to compartmentalize signaling pathways, by bringing appropriate signaling molecules within close-proximity, allowing for efficient signal transduction. This idea is supported by the fact that by blocking internalization of K1, K1 was unable to activate Akt. This is also reinforced by the intracellular co-localization of K1 and the p85 subunit of PI3K. By inhibiting K1 from internalizing, the ability of K1 to efficiently interact with the molecules required to propagate signal transduction is disrupted (Figure 1). This is plausible, as it has been shown that some cellular receptors require correct internalization and trafficking in order to transmit accurate signals. Extensive studies involving the epidermal growth factor receptor (EGFR) have shown the need for compartmentalization for efficient signal transduction (28, 31, 32).

We also analyzed K1 internalization in B lymphocytes, as it relates to the B-cell Receptor (BCR). K1 inhibits BCR assembly in the endoplasmic reticulum, resulting in a decrease in surface expression of the BCR (14). We were interested in exploring whether K1 protein expressed at the plasma membrane interacted with surface BCR and the consequence of such an interaction. By confocal microscopy, we were able to see that K1 and the BCR internalize together, into vesicle-like structures. The two mutants of K1, which internalize at a much slower rate, appear to retain the BCR on the surface. It is likely that the amino-terminal portion or transmembrane domain of K1 binds BCR since the C-terminal deletion mutant of K1 can still interact with BCR. This suggests another physiological function of K1, which is to scavenge BCR from the surface of B-cells, allowing for additional modulation of BCR signaling function.

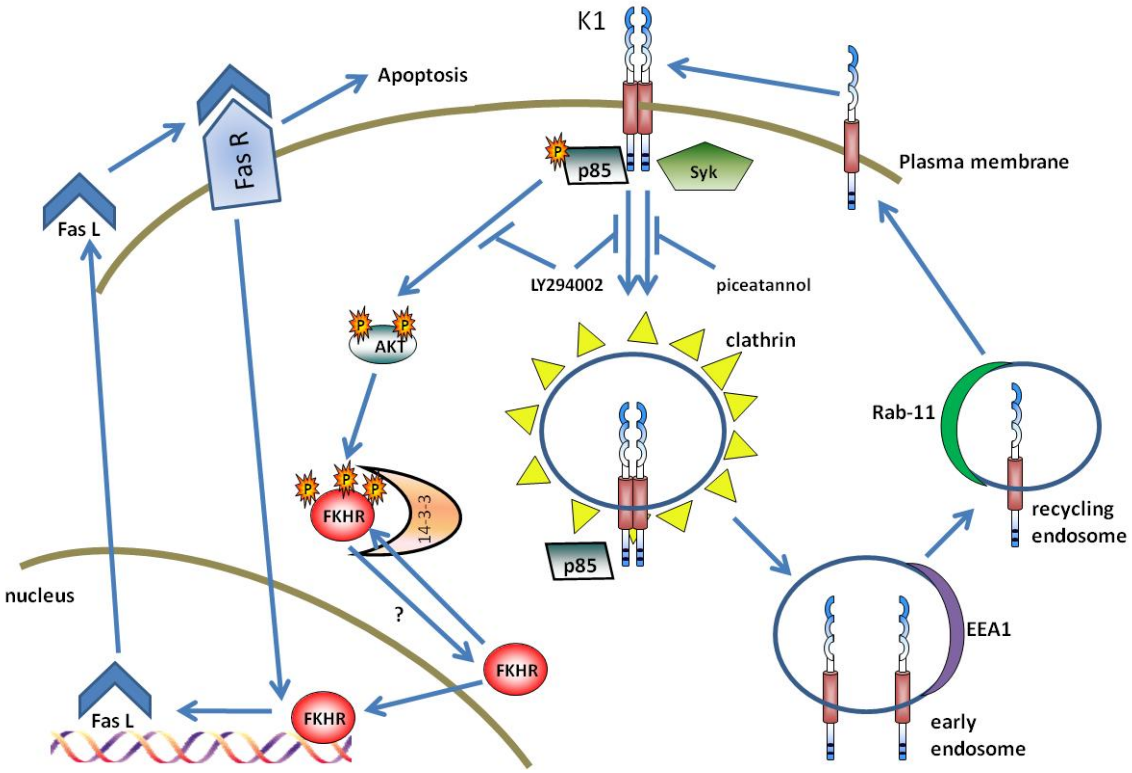

Figure 1. Model of K1 signaling and endocytosis. K1 is constitutively active on the surface of cells via oligomerization of extracellular cysteine residues. K1 activation results in the recruitment of the p85 subunit of PI3K to the plasma membrane. PI3K activation results in the activation and phosphorylation of Akt, which in turn phosphorylates FKHR. Phosphorylation of FKHR causes it to bind to 14-3-3, preventing it from translocating to the nucleus. Upon dephosphorylation, FKHR moves to the nucleus and up-regulates the transcription of Fas ligand, which binds to the Fas receptor on the cell surface, creating a positive feed-back loop, resulting in apoptosis. Therefore, by activating the Akt pathway, apoptosis is inhibited in K1 expressing cells. Activation of K1 causes it to be internalized in a clathrin-dependent manner. Internalization of K1 can be prevented by inhibiting K1 signaling with LY294002 and piceatannol. Once internalized, K1 traffics from the early endosome to the recycling endosome. Trafficking through the recycling endosome allows K1 to be expressed on the surface again, without *de novo* transcription and translation.

Whether or not K1 actually modulates the internalization of the BCR remains to be elucidated. It has been shown that internalization and trafficking to endosomes is not required to amplify BCR signaling, reflecting the dual function of the BCR for both signaling and antigen delivery. Evidence suggests that the BCR remains on the surface to signal, as inhibiting internalization of the BCR actually increases down-stream signaling (34). Internalization may functions to dampen signaling and target antigens for processing for presentation. K1 has not been shown to target to lipid rafts, and does not associate with caveolin, suggesting that by internalizing with BCR, K1 prevents BCR signaling. Normally, cross-linking the BCR accelerates trafficking through the endocytic pathway to specialized late endosomal compartments rich in MHC class II (MIIC) (33). Whether K1 influences the lysosomal degradation of BCR, or the ability of BCR to interact with MIIC is not known and further studies are needed in order to further define the final destination of K1/BCR complexes. The multifaceted interaction of BCR sorting, interaction with MIIC and subsequent antigen presentation will make determination of K1 effects difficult; nonetheless such experiments will give insight into additional means by which KSHV modulates the host immune response.

In chapter four, we turned to a model system for KSHV, Rhesus Monkey Rhadinovirus (RRV). The RRV R1 gene is a homolog of K1. We wanted to determine K1's role in the viral lifecycle in vivo. Since KSHV does not infect mice or macaques, the only available model system to study K1's role in viral pathogenesis is the RRV system. Hence, we decided to delete R1 from RRV and examine the properties of the RRVΔR1 recombinant virus. In order to delete R1 from the RRV genome, we utilized a set of overlapping cosmids encompassing the RRV

genome. We inserted a GFP-expression cassette into the R1 ORF of cosmid ah28ΔA/H thereby interrupting the R1 ORF.

We have analyzed this virus, RRVΔR1-GFP$_{CC}$, for genome integrity. By PCR and Southern blot, RRVΔR1-GFP$_{CC}$ appears to be identical to wild-type RRV H26-95, except for the insertion of the transgene. We next analyzed the virus for replication in a one-step growth curve. We compared RRVΔR1-GFP$_{CC}$ growth to several other viruses: (i) RRV-GFP$_{CC}$, a GFP-expressing RRV also made using the cosmid system, (ii) rRRV-2.3.1, a revertant virus of RRVΔR1-GFP$_{CC}$ in which the GFP expression cassette has been removed, and (iii) RRV-J, a wild-type RRV H26-95 derived from the cosmid system. Preliminary data using real-time PCR based viral load assays showed no significant differences in growth between RRVΔR1-GFP$_{CC}$ and RRV-GFP$_{CC}$. However, there does appear to be an approximate one-log difference between the two wild-type viruses, RRV-J and rRRV-2.3.1. Technically these two viruses should be the same. Thus it may be necessary to further analyze the genomic integrity of both viruses. Confirmation of replication by plaque assay, which measures infectious virus, will also be performed. A plaque assay measure infectious virus while the real time PCR viral load assay measures genome copy numbers. Thus, it is possible that there will be a difference between WT RRV and RRVΔR1-GFP$_{CC}$ infectious titers as measured by plaque assay although the genome copy numbers between these two viruses may be unchanged. This would suggest that R1 may play a role in efficient packaging of infectious virion progeny.

The above experiment was performed at an MOI=0.1. We will also analyze these viruses at differing MOI's. In data not shown, we did see an approximate one fold increase in replication of RRVΔR1-GFP$_{CC}$ over RRV-GFP$_{CC}$, as measured by both real-time PCR and

plaque assay, at an MOI=1.0. Comparing this to the other control viruses will need to be performed, as RRV-GFP$_{CC}$ may not be the appropriate comparison.

For K1, there are contrasting reports as to its effect on lytic replication. Lagunoff *et al* found that expression of K1 in BCBL-1 cells augments lytic replication (13). Conversely, Lee *et al* found that expression of K1 in the same cells resulted in a dramatic decrease in lytic replication (15). However, these two groups induced lytic replication by different means. Lagunoff used exogenous expression of RTA and Lee used TPA. Interestingly, Lagunoff found that ITAM mutants of K1 blocked lytic replication; this block could be overcome with TPA, suggesting that signaling pathways activated by TPA could compensate for the lack of signaling by K1 ITAM mutants. They did not however, show what effect TPA had when used in conjunction with wild-type K1 expression. It is likely that the signaling pathways activated by TPA are similar to that of K1. K1 could be depleting the intracellular pools of signaling molecules used in TPA signal transduction. Lee also found that full-length, wild-type K1 required antibody cross-linking in order to suppress TPA-mediated lytic replication. Antibody cross-linking could result in an increase in K1 signaling transduction. This supports a role for K1 signaling competing for signaling molecules needed for TPA signal transduction. RTA and TPA induce lytic reactivation by different means. TPA induces the activation of several signaling pathways, including PKCγ and ERK, which leads to activation of AP-1, a transcription factor that is required for RTA promoter activation (6). In support of this hypothesis, it has been shown that K1 expression leads to a decrease in RTA expression (15).

Determining the effect that R1 has on *de novo* lytic replication will shed light on the role of K1 in KSHV. We have also created a panel of RhF stable cell lines expressing different R1

C-terminal mutants. Using these complementing cell lines and our RRVΔR1-GFP$_{CC}$ virus, we will analyze the function of the different domains of the R1 C-terminus in RRV lytic replication.

In addition to determining the role R1 plays in lytic replication, we will also analyze the role R1 plays in the establishment of latency and reactivation from latency. We have generated a number of different cell lines infected with RRVΔR1-GFP$_{CC}$ and RRV-GFP$_{CC}$. Using these cells, we are able to analyze infection, based on GFP expression. We will also be able to assess reactivation, by both TPA and RRV-RTA expression and analyze virus progeny by both real time PCR and plaque assay.

These viruses also give us a means to analyze the contribution of R1 to disease *in vivo.* It has been shown in the context of SIV co-infection, that experimental infection with wild-type RRV, results in B cell hyperplasia, persistent lymphadenopathy, and persistent infection in macaques (9, 17, 41). As R1 has been shown to deregulate signal transduction in B-cells (7), R1 may play an important role in the development of disease *in vivo,* perhaps in the context of the development of B-cell hyperplasia.

K1 and R1 play a specific role in the complex interaction between virus and cell. Deregulation of cellular signaling pathways involved in the infection process and replication of virus likely contribute to pathogenesis and viral oncogenesis. By interfering with signaling cascades, KSHV can manipulate not only its own gene expression, but also host gene expression to create a permissive environment for virus infection. By determining how K1 and R1 function in the viral life-cycle, we may shed light on future treatments, not only for KSHV-related diseases, but other neoplasms, as well.

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