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MOLECULAR GENETICS AND FUNCTIONS OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS GLYCOPROTEINS IN VIRAL ENTRY AND VIRUS-INDUCED CELL FUSION

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by
Rafael E. Luna
B.S. Southern University, 1995
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I sincerely thank all the members of my family; they have all aided me in my quest to become a scientist. I also would like to thank my scientific family, BioMMED. I thank all of the members of my laboratory for the camaraderie we shared, which will be forever cherished in my heart; I will miss them deeply. Special thanks are due to my major professor Konstantin G. Kousoulas, Ph.D. for his undying commitment to my development as a professional and compassionate individual. I am extremely grateful to all of my friends who have supported me throughout my graduate studies. I attribute my success, in part, to the inquisitive atmosphere constantly present along the halls of the third floor of the Veterinary Medicine Building.
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ABSTRACT

Kaposi’s sarcoma-associated herpesvirus (KSHV) is considered the etiologic agent of Kaposi’s sarcoma and several lymphoproliferative disorders. Recently, the full-length KSHV genome has been cloned into a bacterial artificial chromosome (BAC) and successfully recovered in 293 cells. The herpesviral glycoproteins are structural components of the KSHV particle and are thought to facilitate virus entry, egress and virus-induced cell fusion. Investigations, described herein, have focused on the genetic manipulation of the KSHV-BAC36 in order to address the role of K8.1 glycoprotein in virus entry and the role of the carboxyl tail α-helices of glycoprotein (gB) in virus-induced cell fusion. In addition, a panel of KSHV-glycoprotein mutants was constructed in order to address the specific role of each glycoprotein in viral entry, egress and virus-induced cell fusion. To further address the role of K8.1 in virus infectivity, a K8.1-null recombinant virus (BAC36ΔK8.1) was constructed by deletion of most of the K8.1 open reading frame and the insertion of a kanamycin resistance gene cassette within the K8.1 gene. Transfection of the mutant genome (BAC36ΔK8.1) DNAs into 293 cells produced infectious virions in the supernatants of transfected cells. Hence, these results clearly demonstrated that the K8.1 glycoprotein is not required for KSHV entry into 293 cells. In addition, two recombinant BAC36-derived genomes were constructed (via a two-step homologous recombination procedure in bacteria) specifying truncations that fully or partially truncated a predicted α-helical structure of the gB carboxyl terminus known to be involved in virus-induced cell fusion from studies with the herpes simplex virus type 1 (HSV-1) gB. Initial experiments suggested that disruption of the predicted α-helical structure gBtH2 enhanced virus-induced cell fusion. Furthermore, utilization of the
pGET-Rec bacterial recombination system for insertional/deletional mutagenesis of the KSHV-BAC36 genome was successfully performed to produce the following KSHV-BAC36 null mutants: KSHV-BAC36ΔgB, KSHV-BAC36ΔgH, KSHV-BAC36ΔK8.1, KSHV-BAC36ΔgL and KSHV-BAC36ΔgM. The investigations herein have capitalized on the recent development of the KSHV-BAC36 clone in order to deliver targeted mutations to the KSHV genome.
CHAPTER I
INTRODUCTION

STATEMENT OF RESEARCH PROBLEM AND HYPOTHESIS

Kaposi’s Sarcoma Associated Herpesvirus (KSHV) is the most recently discovered human herpesvirus (HHV-8). KSHV has been unequivocally linked to three malignant and proliferative disorders: Kaposi’s Sarcoma, Body Cavity-Based Lymphomas, and certain variants of the Multicentric Castleman’s Disease. Due to KSHV’s oncogenic potential, there has been intense research interest in the virus, yet certain basic pathogenic mechanisms regarding the viral lifecycle has not been elucidated, particularly the mechanisms of viral entry and egress.

KSHV encodes many glycoproteins, some of which have significant homology to glycoproteins of other viruses within the Herpesviridae Family; these include the following: gB (ORF 8), gH (ORF 22), gM (ORF 39), gL (ORF 47) (Neipel, Albrecht, and Fleckenstein, 1997; Russo et al., 1996), and gN (ORF 53) (Koyano et al., 2003). In addition, K1, K8.1 and vOX2 (K14) glycoproteins are unique to KSHV with no counterparts in other herpesviruses (Chandran et al., 1998; Chung et al., 2002; Neipel, Albrecht, and Fleckenstein, 1997). KSHV glycoproteins gB and K8.1 mediate initial binding of virions onto glycosaminoglycans, e.g. heparan sulfate on cell surfaces (Akula et al., 2001a; Akula et al., 2001b; Birkmann et al., 2001; Wang et al., 2001a). Furthermore, a soluble form of K8.1A inhibited KSHV attachment onto cells (Zhu et al., 1999). However, a later report indicated that a similar soluble form of K8.1A did not block KSHV infectivity (Birkmann et al., 2001). In addition, gB binds to integrins, such
as α3β1 membrane receptors through a RGD motif, suggesting that integrins function as cellular receptors for KSHV entry (Akula et al., 2002; Naranatt et al., 2003). However, soluble integrins or RGD-containing peptides failed to inhibit virus entry into 293 cells (Inoue et al., 2003). In addition to the known role of gB in cell-surface binding, KSHV gB is thought to play important roles in membrane fusion during virus entry (fusion of the viral envelope with cellular membranes) virus-induced cell-to-cell fusion (Baghian et al., 1993; Foster, Melancon, and Kousoulas, 2001), functions that seem to be conserved for all gB homologues specified by all human and animal herpesviruses.

Recently, it has been shown that infectious virions could be produced using a bacterial artificial chromosome (BAC) containing the entire KSHV genome (Zhou et al., 2002). To facilitate addressing the role of individual glycoproteins in viral entry or egress, a panel of KSHV glycoprotein knockout mutant viruses was produced in bacteria utilizing the KSHV-BAC36 as a viral genomic template. The overall experimental approach was validated by focusing on the role of KSHV K8.1 glycoprotein in virus entry. The specific hypothesis for these investigations was that K8.1 is dispensable for virus entry; this work is described in chapter II. A second aspect of the work focused on gB, which is known to mediate fusion of the viral envelope with cellular membranes during virus entry as well as fusion among cellular plasma membranes for a number of different herpesviruses. gB specific mutant viruses were constructed, containing specific gB mutations which would increase virus-induced cell membrane fusion; this work is described in chapter III. Additional glycoprotein gene deletion mutant genomes were produced for glycoproteins gB, gH, gL and gM; however, due to time constraints these mutants were not fully characterized (described in chapter IV).
STATEMENT OF RESEARCH OBJECTIVES

The overall goal of this research was to gain a better understanding of the role that major viral glycoproteins play in the KSHV lifecycle through the production and characterization of mutant viruses that contained deletions or mutations within each glycoprotein gene. The specific objectives were the following: 1.) To address the role of K8.1 in virus entry and egress; 2.) to address the potential role of the gB carboxyl terminus in virus-induced cell fusion.

LITERATURE REVIEW

Historical Perspective of Kaposi’s-Sarcoma Associated Herpesvirus

In 1872, Moritz Kaposi, a Hungarian dermatologist, was the first to clinically diagnose an aggressive tumor in elderly male patients as idiopathic multiple pigmented sarcomas of the skin (Kaposi 1872). The patients that were originally described in Kaposi’s study eventually died of their condition, which is now referred to as “classic” Kaposi’s Sarcoma (KS). Subsequent to Kaposi’s initial description of this condition, three additional forms of Kaposi’s Sarcoma have also been described: African-endemic, iatrogenic and AIDS-KS. In 1994, a landmark study was published utilizing representational differential analysis to detect the presence of herpesvirus-like DNA sequences in biopsies of AIDS-KS tissue but not in biopsies of non-KS tissue from AIDS patients; thus marking the discovery of a new herpesvirus, which was appropriately named the kaposi’s sarcoma-associated herpesvirus or human herpesvirus 8(Chang et al., 1994). Kaposi’s sarcoma-associated herpesvirus (KSHV) has also been associated with primary effusion lymphomas (PEL), multicentric Castleman’s disease (MCD) and primary pulmonary hypertension.
Herpesviruses are among the oldest viruses known to afflict mankind (Knipe, 2001). KSHV has been taxonomically categorized into the herpesviridae family and the gammaherpesvirinae subfamily. KSHV has been shown to infect a myriad of cells in vitro: endothelial, human foreskin fibroblast, Vero, 293, HeLa cells, etc. KSHV has generated a considerable amount of interest in the scientific community due to the presence of many cellular homologs encoded within its viral genome which allows the virus to persist and evade the host immune system.

Clinical Significance of Kaposi’s Sarcoma-Associated Viruses

Kaposi’s Sarcoma

Kaposi’s Sarcoma is characterized by spindle cells that are undergoing proliferation subsequently forming irregular microvascular channels. Although KS tumors are predominately found in the dermis, tumors have also been found in the viscera (Hengge et al., 2002) of some patients. Visceral dissemination results in organ failure and eventual death. Currently there are four clinical forms of KS with varying epidemiological parameters, which are listed as followed: 1.) Classic, 2.) AIDS-associated (epidemic), 3.) African (endemic), and 4.) Iatrogenic. The underlying current among all of the clinical variants of KS is the requisite infection of the host by KSHV(Aluigi et al., 1996; Ambroziak et al., 1995; Foreman et al., 1997a; Gao et al., 1996b; Kedes et al., 1996; Lennette, Blackbourn, and Levy, 1996; Miller et al., 1996; Parravicini et al., 1997b; Qunibi et al., 1998; Regamey et al., 1998; Schalling et al., 1995; Simpson et al., 1996). KSHV seroconversion has been shown to occur before the manifestation of KS and could be used to predict the development of disease(Cesarman et al., 1996; Gao et al., 1996a; Kedes et al., 1996; Martin et al., 1998; Renwick et al., 1998).
The assortment of the clinical variants of KS along with the other lymphoproliferative disorders associated with KSHV infection underscores a substantial role for cofactors in KS pathogenesis (Dourmishev et al., 2003)

**Classic KS** appears as rare indolent tumors occurring in elderly men of Mediterranean descent (Ablashi et al., 2002; Dourmishev et al., 2003; Moore and Chang, 2003). The classic KS tumors manifest themselves in the lower extremities for approximately ten or more years with a very low mortality rate (Hengge et al., 2002). In the late 1800s, Moritz Kaposi was the first individual to describe the clinical condition of “classic” KS in various patients; he described the patients as having multifocal pigmented sarcomas (Kaposi, 1872). Those initial patients died of KS, yet the clinical presentation of the disease is grossly similar to what we currently see in KS patients (Knipe et al., 2001).

**Epidemic AIDS KS** served as a harbinger of the AIDS complex for homosexual men in the early 1980s (Borkovic and Schwartz, 1981; Gottlieb et al., 1981; Hymes et al., 1981); these individuals were dually infected with HIV and KSHV. Due to the concurrent infection and synchronized epidemics of both AIDS and KS, this particular epidemiological form of KS was termed epidemic AIDS KS (Borkovic and Schwartz, 1981; Gottlieb et al., 1981; Hymes et al., 1981). The most prevalent neoplasm in homosexual AIDS patients has been KS, the AIDS epidemic form (Goedert, 2000). Serological assays indicate a marked increase of KSHV prevalence in AIDS patients before the onset of KS (Gao et al., 1996b; Martin et al., 1998; Simpson et al., 1996).

HIV and KSHV dually infected individuals provide a milieu which allows the augmentation of KSHV pathogenesis by HIV at various stages: immunosuppression,
enhancement of KSHV infection and replication, and alteration of the KSHV gene expression profile (Dourmishev et al., 2003). The most pronounced risk factor for AIDS-associated KS in individuals infected with HIV is sexual behavior (Martin et al., 1998). HIV infected homosexuals have a more than ten thousand time risk of developing KS as opposed to individuals in the general population (Goedert, 2000). There is mounting evidence that orogenital sex as opposed to anogenital sex is a greater risk factor for KSHV infection (Dukers et al., 2000). The primary mode of KSHV transmission has yet to be determined; however, recent evidence has postulated that KSHV transmission via the oral route may also occur among healthy immunocompetent individuals (Cook et al., 2002a; Cook et al., 2002b; Duus et al., 2004). A retrospective study, analyzing the prevalence of KSHV infection among homosexual men at the beginning and during the AIDS epidemic, showed that unprotected oral sex exhibited the highest behavioral risk factor (Osmond et al., 2002). KSHV has been readily detectable in the saliva of seropositive individuals (Blackbourn et al., 2000; Koelle et al., 1997; Pauk et al., 2000; Stamey et al., 2001; Vieira et al., 1997), yet the detection of KSHV in semen has been extremely controversial with very low reproducibility. Thus, the ever increasing evidence suggests differing routes of transmission for KSHV and HIV.

**Endemic Africa KS** has preceded the widespread HIV pandemic for many decades in Africa, particularly the equatorial region (Oettle, 1962). The greatest number of African KS patients were found in Zaire, Uganda, and Tanzania (Ablashi et al., 2002). Before the outbreak of HIV, African KS primarily affected young children of an approximate age of three years; African KS also affected men of an approximate age of thirty-five years (Wabinga et al., 1993).
Microparticles of silica dust have been mentioned as an environmental cofactor in children with African KS (Ziegler, Simonart, and Snoeck, 2001). Intralymphatic silica, from animal models involving the direct intralymphatic injection of fine silica particles, incited a rapid and robust macrophage reaction with subsequent fibrosis within lymph vessels (Fyfe and Price, 1985). Theoretically, children who walk barefoot risk the injection of microparticles of silica at the site of their feet, thus perhaps leading to immune suppression in a localized area, which provides the proper milieu for the development of African KS in individuals infected with KSHV (Ablashi et al., 2002). African KS typically exhibits a more aggressive clinical manifestation as opposed to classic KS, yet African KS presents a less fulminant clinical course in comparison to African AIDS-associated KS (Bayley, 1984; Wabinga et al., 1993).

Due to the widespread dissemination of AIDS on the continent of Africa, KS is the most frequently reported neoplasm in several African countries, which constitutes a severe public health dilemma that has yet to be addressed (Bassett et al., 1995; Beral, Newton, and Sitans, 1999). Epidemiologic studies differentiating between endemic African KS and AIDS-KS would be impractical in various African countries. Although the prevalence of KS has exploded in the HIV positive communities in Africa, a marked increase in the prevalence of KS has been noticed in the HIV negative community, which could be referred to as the modern version of endemic African KS (Ablashi et al., 2002).

Iatrogenic (transplant) KS has become a growing concern for transplant recipients. KSHV has been shown to be epidemiologically linked to KS in post-transplant patients, particularly patients with kidney or liver transplants (Luppi et al., 2003). In comparison to lymphomas or various epithelial malignancies, iatrogenic KS
presents itself as an earlier manifestation of post-transplantation malignancy in transplant patients (Penn, 1993). Transplant patients with KS typically involve skin lesions; however, a significant portion of patients manifest visceral dissemination of KS (Singh, 2000). Remission of KS has been reported in all variants of KS upon removal of immunosuppression; however, iatrogenic KS patients need to be immunosuppressed in order to prevent allograft rejection. Greater than fifty percent of iatrogenic KS patients have experienced tissue rejection upon removal of immunosuppression (Singh, 2000). There has been a contention of whether or not KS in posttransplant patients is due to reactivation of KSHV or due to KSHV primary infection via infected organ-donor tissue. Furthermore, it has been postulated that iatrogenic KS is due to KSHV reactivation in areas of endemic KS; however, in areas of nonendemic KS, it is believed that post-transplantation KS is due to primary infection (Rabkin, Shepherd, and Wade, 1999). Accumulating evidence from several published papers suggests that KSHV primary infection of the patient is significantly higher than previously expected (Andreoni et al., 2001; Cattani et al., 2001; Emond et al., 2002; Farge et al., 1999; Kapelushnik et al., 2001; Luppi et al., 2000a; Luppi et al., 2000b; Munoz et al., 2002; Sarid et al., 2001). Thus, donor/recipient screening measures, which assay for KSHV positivity, could theoretically alleviate the number of new KSHV primary infections or reactivations in recipient patients via the assessment of high risk patients and modulation of their immunosuppression to reduce the possibility of developing KS (Cannon, Laney, and Pellett, 2003; Luppi et al., 2003).
**Pleural Effusion Lymphoma (PEL)**

As opposed to KS, Pleural effusion lymphomas (PELs) manifest the more common neoplastic proliferation process, in which transformed cells exhibit a dysregulated cellular expansion (Knipe et al., 2001). PELs, formerly known as Body Cavity Based Lymphomas, was described first in AIDS patients (Cesarman et al., 1995). PELs are extremely rare yet could be found predominately in HIV infected patients, but not exclusively; PELs exhibit distinct clinical, immunophenotypic, and molecular characteristics (Aoki and Tosato, 2003; Knipe et al., 2001). The malignancy presents itself as an aggressive effusion in the peritoneal, pericardial, pleural, or abdominal cavity, typically without an identifiable tumor mass (Aoki and Tosato, 2003; Leao et al., 2002). In rare cases, PELs manifest as solid tumor masses outside the body cavities (Aoki and Tosato, 2003; Leao et al., 2002). The prognosis for AIDS patients diagnosed with PEL is extremely poor; usually development of the PEL malignancy would lead to death within an average of six to eleven months on standard chemotherapy (Kaplan et al., 1997; Levine et al., 1991). PELs have been found to be useful in scientific research to derive cell lines which provide a source of KSHV production readily used in virologic and serologic assays; PELs harbor approximately fifty to one-hundred and fifty viral episomes per cell (Moore and Chang, 2003). Typically, most of the KSHV genomes are harbored in the PEL cells in a latent state; however, KSHV within a small percentage (less than three percent) of PEL cells spontaneously undergo lytic reactivation (Renne et al., 1996b). Induction of the viral lytic cycle could be achieved by the addition of a phorbol ester, tetradecanoyl-13-myristate acid or TPA (Renne et al., 1996a; Renne et al., 1996b; Sarid et al., 1998; Zhong et al., 1996).
Investigators agree that PELs are derived from a B-cell origin, late-stage differentiated both pre- and post-germinal center B cells, thus PELs are not restricted to any stage of B cell maturation (Carbone et al., 1998; Matolcsy, 1999). Another study has shown that KSHV is detected in circulating B cells (Blackbourn et al., 1997). Infection of B cells by KSHV mirrors the infectivity of cells susceptible to the Epstein-Barr virus (EBV); a virus that also transforms cells and causes lymphoproliferative disorders in infected patients (Knipe et al., 2001). Most PELs are dually infected with KSHV and EBV genomes; the precise concerted viral mechanisms employed by both viruses leading to the lymphoproliferative effusions are not fully understood (Ablashi et al., 2002).

**Multicentric Castleman’s Disease (MCD)**

Multicentric Castleman’s Disease (MCD), which is also named multicentric angiofollicular lymphoid hyperplasia, in its systemic proliferative form involves fever, lymphadenopathy, splenomegaly (Mikala et al., 1999). MCD could be readily characterized by vascular proliferation in the germinal centers, which in this regard is similar to KS (Ablashi et al., 2002). This rare B-cell lymphoproliferative disorder is found mostly in HIV infected AIDS patients (Aoki and Tosato, 2003; Leao et al., 2002). The predominate number of cases of MCD (greater than 90%) in AIDS patients are infected with KSHV, thus suggesting a robust role of KSHV in the pathogenesis of MCD, particularly in AIDS patients (Grandadam et al., 1997). Furthermore, in AIDS related MCD, KSHV viral loads are high even though there is a lack of manifest MCD foci within the lymph nodes of the infected patient (Parravicini et al., 1997a). In HIV-negative healthy individuals, fifty percent of all individuals with MCD are infected with KSHV;
however, the etiologic factors of the remainder of MCD patients that are KSHV negative are unclear (Moore and Chang, 2003).

MCD could be categorized into either a localized and systemic manifestation. Analogous to KS, MCD patients dually infected with HIV and KSHV present a significantly more aggressive form of MCD with a concomitant poor prognosis (Oksenhendler et al., 2000; Parravicini et al., 1997a; Zietz et al., 1999). In regard to KSHV infection, MCD is a polyclonal tumor wherein the majority of the cell mass of uninfected lymphocytes are recruited by KSHV infected B cells (Dupin et al., 1999; Katano et al., 2000; Parravicini et al., 1997a). As opposed to PELs wherein most PEL cells are dually infected with KSHV and EBV, MCD usually lacks co-infection with EBV (Aoki, Jones, and Tosato, 2000; Little et al., 2001). The proportion of KSHV lytically infected to latently infected cells is significantly higher in MCD as opposed to PEL, thus implying different KSHV pathogenetic mechanisms found in MCD as opposed to PEL, which could be ascribed to varying cellular and viral gene expression profiles in both of these lymphoproliferative disorders (Rivas et al., 2001; Staskus et al., 1999; Teruya-Feldstein et al., 1998).

**Structure of the KSHV Particle**

Structural studies have shown that KSHV manifests similar structural features as the other members of the *Herpesviridae* family. KSHV possesses the typical morphological characteristics with an approximately 100-150nm observed size of the virion particle (Renne et al., 1996b). All herpesviruses are comprised of four structural elements: 1.) a cylindrical core structure wherein linear double stranded DNA is present; 2.) an icosahedral capsid consisting of 162 hexagonal capsomeres, 3.) an amorphous
tegment which surrounds the capsid, and 4.) a lipid bilayer envelope which is derived from the host cell (Knipe et al., 2001).

Virion DNA extracted from the phorbol ester (12-O-tetradacanoyl-13-acetate; TPA) induced BCBL-1 cells revealed that the KSHV genome is comprised of approximately 165kb to 170kb (Renne et al., 1996a; Zhong et al., 1996). The KSHV genome size is a bit larger than the prototypical herpesvirus, HSV-1, which is 135 kb. Yet, the genome size is considerably smaller in size when compared to the viral genome of the human cytomegalovirus, (HCMV). Although numerous studies have been published detailing KSHV infection and pathogenesis, there has been a dearth of studies addressing virion structure and morphology. KSHV has been difficult to cultivate in either tissue culture or animal systems. It has been shown that KSHV and HCMV capsids are similar via negative stained images of thin sections; KSHV nucleocapsids was shown to be approximately 120-150nm, while HMCV was shown to be 150-200nm (Said et al., 1997). Using electron cryomicroscopy, investigators have shown that the capsid size and structure accords well with other members of the herpesvirus family (Trus et al., 2001; Wu et al., 2000).

**The Core and Organization of the Viral Genome**

The innermost core of the KSHV particle is similar to all other herpesviruses in which the viral DNA genome is wound (Knipe et al., 2001). Herpesviruses possess a linear double-stranded DNA genome, which range in size from 120kb to 250kb. The KSHV genome is approximately 165 to 170kb, as shown by pulse-field gel electrophoresis of DNA purified from intact virions (Renne et al., 1996a). Nucleotide sequence data indicated that KSHV belongs to the gammaherpesvirus subfamily, genus *Rhadinovirus* (gamma-2 herpesvirus);
KSHV is the first member of this genus known to infect humans (Moore et al., 1996b). The linear double stranded genome consists of a 140.5kb central segment of low-GC DNA [also known as L DNA or long unique region (LUR)], which has 53.5% G+C content (Moore et al., 1996b; Neipel, Albrecht, and Fleckenstein, 1997; Russo et al., 1996). The LUR is flanked by 20-35kb multirepetitive high GC DNA, which has 84.5% G+C content [also known as H DNA or terminal repeats (TR)] (Moore et al., 1996b; Neipel, Albrecht, and Fleckenstein, 1997; Russo et al., 1996).

Approximately 90 genes have been identified within KSHV’s LUR, which exhibits similar genetic organization to herpesvirus saimiri (HVS) (Moore et al., 1996b; Neipel, Albrecht, and Fleckenstein, 1997). KSHV and HVS share approximately 70 conserved genes that are arranged co-linearly, with small intermittent regions of genes unique to each virus. The nomenclature for individual viral genes corresponds to the previous assignation of genes for HVS, thus each conserved gene has been designated the prefix “ORF” and numbered consecutively from left to right across the viral genome, while the KSHV idiosyncratic genes within the intermittent regions have been named K1 to K15 (Russo et al., 1996). Other nonhuman members of the genus rhadinovirus, such as the rhesus rhadinovirus and murine gammaherpesvirus 68, have shown stark conservation regarding their genetic organization.

The conserved genes among the rhadinovirus genus are those which function in both virion structure and viral DNA replication (in either metabolic or catalytic roles) such as the following: glycoprotein B (ORF8), glycoprotein H (ORF22), DNA polymerase (ORF9), polymerase processivity factor (ORF59), DNA helicase-primase complex (ORF40, ORF41 and ORF44), thymidylate synthase (ORF70), and thymidine
kinase (ORF21) (McGeoch and Davison, 1999; Moore et al., 1996b; Neipel, Albrecht, and Fleckenstein, 1997; Russo et al., 1996). Interestingly, KSHV and the Epstein-Barr virus (EBV), the only other human gammaherpesvirus, share both structural and biological features with a lucid genetic correspondence; both KSHV and EBV are considered oncogenic viruses yet KSHV lacks any of the EBV unique latency genes (Knipe et al., 2001; Russo et al., 1996). EBV and KSHV both utilize B cells as a reservoir during latency and they both cause B cell tumors (Knipe et al., 2001). EBV proteins induce the expression of a certain set of cellular genes, and KSHV seems to have captured and modified this set of human cDNAs and incorporated them into its genome (Knipe et al., 2001; Neipel, Albrecht, and Fleckenstein, 1997).

**The Capsid**

Similar to other herpesviruses, capsids are the first structures that arise after the initiation of KSHV replication, which accumulate in the nucleus and upon further maturation the capsids harbor the linear viral genome. However, viral DNA packaging only occurs in a fraction of newly synthesized capsids (Knipe et al., 2001; Renne et al., 1996b). Encapsidated KSHV DNA is thought to be analogous to encapsidated HSV-1 DNA, which is present within the capsid as a single linear copy encoding the entire viral genome (Booy et al., 1991). In comparison to EBV, the KSHV genome is present in infected cells as a circular form during latency and upon reactivation the viral genome linearizes and is amenable to subsequent incorporation into capsids (Decker et al., 1996; Knipe et al., 2001; Renne et al., 1996a).

In cells infected with a herpesvirus, lytic replication leads to the accumulation of viral capsids within the nuclei, thus investigations of HSV-1 and HCMV capsid
structures have used the nuclei of infected cells as a source of viral capsids for biochemical analyses (Homa and Brown, 1997). Unfortunately, structural capsid studies of gammaherpesviruses have been hampered by the unsuccessful attempts of procuring a sufficient amount of stable intranuclear capsids. For EBV and KSHV, this technical hurdle was overcome by using released virions in the supernatants of induced infected cells as a stable source of KSHV capsids (Dolyniuk, Pritchett, and Kieff, 1976; Nealon et al., 2001; Trus et al., 2001; Wu et al., 2000). The establishment of a stable source of KSHV capsids has allowed investigators to determine the protein composition of the capsid and the molecular ratios of the proteins involved in the architecture of the capsid (Nealon et al., 2001; Trus et al., 2001; Wu et al., 2000). KSHV infected cells produce three major capsid types, which can be easily visualized via electron microscopy and separated by density gradient sedimentation. The three major capsid forms are the following: 1.) A capsids, 2.) B capsids and 3.) C capsids. A capsids, which have a total mass of 200MDa, are empty icosahedral shells void of DNA or any detectable internal structure. B capsids, which have a total mass of 230 MDa, are icosahedral shells filled with an inner array of scaffolding protein originated from ORF17.5 (Nealon et al., 2001). C capsids, which have a total mass of 300 MDa, are icosahedral shells that are filled with one copy of the KSHV DNA yet lack the scaffolding protein, which was present in the B capsids. The A, B, and C capsid types consist of four viral proteins the ORF25/MCP (major capsid protein), ORF62/TRI-1 (triplex-1), ORF26/TRI-2 (triplex-2), and ORF65/SCIP (small capsomer interacting protein) (Nealon et al., 2001; Trus et al., 2001; Wu et al., 2000). Herpesvirus capsids consist of 150 hexameric and 12 pentameric capsomers formed exclusively from the KSHV-ORF25 product, the major capsid protein
(MCP). The majority of the capsid’s mass could be attributed to MCP, which is highly conserved throughout the herpesvirus family (Homa and Brown, 1997). The capsomers are linked together via hetero-triplexes, which protrude from the capsid floor in between the capsomer structures (Trus et al., 2001; Wu et al., 2000). In KSHV, the hetero-triplexes consist of two molecules of ORF26/TRI-2 and one molecule of ORF62/TRI-1 (Nealon et al., 2001). Type C capsids are the only type of capsid isolated by de-enveloping intact virions; moreover, type C capsids are the least abundant capsid type (about 10-15% of total capsids produced) found in the supernatants of induced BCBL-1 cells (Gibson and Roizman 1972; Shrag et al 1989; Nealon et al., 2001). The small percentage of type C capsids produced from BCBL-1 cells may provide an explanation of the low infectivity of KSHV in experiments using this cell line as a source of virus stock (Nealon et al., 2001).

**The Tegument**

Generally, the tegument layer of herpesviruses is an amorphous proteinaceous region between the outer surface of the capsid and the underface of the envelope; the tegument is considered to manifest variations in both size and composition (Roizman and Furlong, 1974). Limited information is available in about the structure and function of the herpesvirus tegument, particularly the KSHV tegument. Certain herpesvirus tegument proteins have been shown to possess regulatory functions essential for the viral lifecycle; tegument proteins are functional upon penetration of the virus into cells. Thus tegument proteins play a key role in creating a conducive milieu for viral replication (Knipe et al., 2001). KSHV ORF45 is an immediate-early protein shown to be present in the tegument area of virions, thus suggesting that KSHV ORF45 plays a role in primary
infection (Zhu and Yuan, 2003). ORF45 protein has been shown to bind cellular IRF-7 and inhibits its phosphorylation and transport from the cytoplasm to the nucleus in response to viral infection (Zhu et al., 2002). The type I IFNs (Interferons) are produced by virus infected cells and is regulated, in part, by IRF-7, (IFN-regulatory factor) a transcription regulator that serves a critical role in virus-mediated induction of IFN-α and IFN-β (Samuel, 2001; Stark et al., 1998). In addition to tegument proteins, mRNAs, spanning more than one kinetic class, are also packaged within infectious virions of alpha and betaherpesviruses (Bresnahan and Shenk, 2000; Sciortino et al., 2001; Sciortino et al., 2002). It remains to be seen whether or not KSHV also possesses mRNAs within intact virions.

The Envelope

A lipid bilayer envelope, obtained from the infected cell, in which viral glycoproteins are embedded, constitutes the outermost layer of the KSHV particle. KSHV specifies several glycoproteins designated as gB, gH, gpK8.1, gL, gM, gN and others (Koyano et al., 2003; Russo et al., 1996). The majority of herpesviral glycoproteins function throughout the viral lifecycle, in the following roles: attachment, penetration, egress, and virus-induced cell fusion (Knipe et al., 2001).

Kaposi’s Sarcoma-Associated Herpesvirus Lifecycle

The principal events in the KSHV lifecycle in cell culture systems involve the attachment of the virus to the cell surface, penetration of the virus into the susceptible cell, host protein synthesis shut-off, movement of the de-enveloped tegument-capsid structure toward the nuclear pores thereby releasing viral DNA into the nucleus, coordinated cascade of viral genome transcription, replication of the viral DNA, and
Attachment

Attachment of many enveloped viruses is known to be mediated by interactions between specific viral glycoproteins with cellular receptors. Typically, herpesvirus entry into a susceptible cell can be categorized into two steps: 1.) Attachment of virus to cell surfaces and 2.) Fusion of the viral envelope with cellular membranes leading to entry of viral capsids into the cellular cytoplasm. Initial virus attachment events provide loosely associated interactions with cells and are mediated via KSHV glycoproteins with ubiquitous heparan sulfate proteoglycan moieties on the surface of susceptible cells (Akula et al., 2001a; Akula et al., 2001b; Birkmann et al., 2001; Wang et al., 2001a).

Proteoglycans consist of a protein core with one or more covalently attached glycosaminoglycans such as heparan sulfate or chondroitin sulfate. Proteoglycans are found abundantly on the surface of mammalian cell membranes, as integral membrane proteins, glycerol phosphatidyl inositol-linked membrane proteins, and proteins of extracellular matrices (Stringer and Gallagher, 1997). Proteoglycans serve in various fundamental cellular processes such as the following: 1.) cell-to-cell adhesion, 2.) cell-to-matrix adhesion, 3.) cellular motility, 4.) cellular growth and 5.) cellular signaling (Kjellen and Lindahl, 1991; Rostand and Esko, 1997).

Enveloped viruses, particularly herpesviruses, exploit the anatomical characteristics of mammalian cell membranes in both their attachment and entry mechanisms. Herpesviruses spanning every subfamily have been shown to bind heparan sulfate during the initial attachment phase of viral entry. The following herpesviruses
bind heparan sulfate: alphaherpesvirus [HSV-1, HSV-2, PRV, and BHV-1] (Flynn and Ryan, 1995; Flynn and Ryan, 1996; Herold et al., 1994; Laquerre et al., 1998; Liang, Babiuk, and Zamb, 1993; Mettenleiter et al., 1990; Shieh and Spear, 1994; Shukla et al., 1999; Shukla and Spear, 2001; Spear, 2004; Spear and Longnecker, 2003; Spear et al., 1992; WuDunn and Spear, 1989), betaherpesviruses [HCMV and HHV-7] (Navarro et al., 1993; Neyts et al., 1992; Secchiero et al., 1997; Skrinicosky et al., 2000), and gammaherpesviruses [BHV-4 and KSHV] (Akula et al., 2001a; Akula et al., 2001b; Birkmann et al., 2001; Vanderplasschen, 1999; Vanderplasschen et al., 1993; Wang et al., 2001a).

Binding of herpesvirus particles to cell surfaces is inhibited by soluble heparin, a molecule similar in structure to heparan sulfate. The significant inhibition of KSHV binding to susceptible cells suggests that KSHV interacts with cell surface heparan sulfate at the attachment stage; however, the inability of heparin to completely inhibit binding and infectivity indicates that additional ligand-receptor interactions are needed to complete the viral entry process (Akula et al., 2001b). From the cadre of glycoproteins on the envelope of the KSHV particle, virion envelope-associated gB and soluble chimeric K8.1 glycoproteins have been shown to exhibit strong affinity for heparin binding, and their binding to cell surfaces could be inhibited by the addition of heparin in a dose-dependent manner(Akula et al., 2001a; Birkmann et al., 2001; Wang et al., 2001a). Thus, indicating an important role for glycoproteins K8.1 and gB in KSHV attachment mediated by heparan sulfate proteoglycan moieties.
**Receptor Facilitated Entry**

KSHV manifests a broad cellular tropism, which could be attributed partially to the weak binding of KSHV to cell surfaces via the ubiquitous heparan sulfate molecules (Akula et al., 2001a; Akula et al., 2001b; Birkmann et al., 2001; Wang et al., 2001a). KSHV has been shown to infect a variety of human cell types such as the following: B, T, endothelial, epithelial, fibroblast, and keratinocyte cells; the nonhuman cell types that are susceptible to KSHV are the following: owl monkey kidney and baby hamster fibroblast cells (Cerimele et al., 2001; Ciufò et al., 2001; Flore et al., 1998; Foreman et al., 1997b; Gao, Deng, and Zhou, 2003; Kliche et al., 1998; Mesri et al., 1996; Moore et al., 1996b; Panyutich, Said, and Miles, 1998; Renne et al., 1998; Vieira et al., 2001; Zhou et al., 2002).

Herpesviruses utilize multiple receptors for binding and entry into cells. For example, HSV-1 can utilize one of three different classes of receptors, which strongly bind to to the viral glycoprotein ligand gD subsequent to heparan sulfate binding by HSV-1 gB or gC (Spear, 2004; Spear, Eisenberg, and Cohen, 2000; Spear and Longnecker, 2003). HSV-1 receptors which bind glycoprotein gD include HVEM (herpesvirus entry mediator), a member of the TNF receptor family; nectin-1 and nectin-2, members of the immunoglobulin superfamily; and specific sites in heparan sulfate generated by glucosaminyl 3-O-sulfotransferases (Spear, 2004; Spear, Eisenberg, and Cohen, 2000; Spear and Longnecker, 2003). HSV-1 glycoproteins gC and gD are conserved among most of the members of the alphaherpesvirus subfamily, but exhibit no recognizable structural homologues in members of the *betaherpesvirinae* and *gammaherpesvirinae* of the *Herpesviridae* family (Knipe et al., 2001; Spear, 2004; Spear,
Eisenberg, and Cohen, 2000; Spear and Longnecker, 2003). However, glycoproteins gB, gH and gL are structurally conserved among all herpesviruses and perhaps possess conserved essential fusogenic roles in viral entry (Knipe et al., 2001; Spear, 2004; Spear, Eisenberg, and Cohen, 2000; Spear and Longnecker, 2003).

KSHV was the first herpesvirus shown to utilize integrin as a cellular receptor for target cells (Akula et al., 2002; Nemerow and Cheresh, 2002). KSHV gB contains an RGD motif which binds specifically to the integrin \( \alpha_3\beta_1 \) receptor (CD49c/29) (Akula et al., 2002; Wang et al., 2003). Binding to integrins is not a common feature of herpesvirus gBs; there has been no other herpesvirus gB that possesses a similar conserved RGD motif. The integrin \( \alpha_3\beta_1 \) is broadly expressed and has been detected on all cells susceptible to infection by HHV-8, including human foreskin fibroblasts and B, epithelial, endothelial, and 293 cells (Akula et al., 2002). Also, a recent study has shown that the Epstein-Barr virus, EBV, implements an integrin-mediated mechanism to infect the basolateral surface of polarized oropharyngeal cells; in this study, the BMRF-2 glycoprotein binds via an RGD motif and facilitates entry via the interactions with either the \( \beta_1 \) integrin or \( \alpha_5\beta_1 \) integrin receptors (Tugizov, Berline, and Palefsky, 2003).

The second phase of herpesviral entry subsequent to initial and secondary attachment is penetration of attached enveloped viruses into the cytosol of the infected cell. Viruses have been shown to penetrate susceptible cells via two routes. The first route of viral entry involves the virion fusing its juxtaposed envelope with the apposing cell membrane and then release of the nucleocapsid with accompanying tegument proteins into the cytosol, typically (but not exclusively) herpesviruses utilize this membrane fusion pathway (Knipe et al., 2001; Liu et al., 2002; Marsh and Pelchen-
Matthews, 2000). The second entry pathway used by viruses is a receptor-mediated endocytosis of virions into susceptible cells; nonenveloped DNA viruses, nonenveloped RNA viruses and enveloped RNA viruses utilize the endocytotic pathway (Anderson, Chen, and Norkin, 1996; Jin et al., 2002; Joki-Korpela et al., 2001; Marjomaki et al., 2002; Selinka, Giroglou, and Sapp, 2002; Sieczkarski and Whittaker, 2002b).

Endocytosis is a particularly attractive entry mechanism for viruses, particularly DNA viruses, due to the fact that endosomes present a quick and expedient manner for virus to traverse the labyrinthine cytoplasm in order to deliver the viral DNA to an area near the nuclear pore, thus allowing DNA viruses to replicate their genomes in the nucleus of infected cells (Marsh and Pelchen-Matthews, 2000; Sieczkarski and Whittaker, 2002a; Whittaker and Helenius, 1998). KSHV has been shown to enter both human B cell line, BJAB, and human foreskin fibroblast cells, HFF, via large endocytotic vesicles (Akula et al., 2003; Akula et al., 2001b). Epstein-Barr virus (EBV) infects two human cell types, B lymphocytes and epithelial cells. Electron microscopic studies have shown that EBV fuses with the lymphoblastoid cell line Raji but is endocytosed into thin-walled non-clathrin-coated vesicles in normal B cells before fusion takes place (Miller and Hutt-Fletcher, 1992; Nemerow and Cooper, 1984). Although KSHV has been shown to enter B cells and HFF cells via an endocytic pathway, the release of capsids into the cytoplasm is thought to involve fusion of the viral envelope with either plasma or endosomal membranes. Furthermore, KSHV glycoprotein induced membrane fusion in a cell-based assay with susceptible cells as targets has been shown to require only gB, gH, and gL in order to induce cell-to-cell fusion, thus implying a role for these glycoproteins as fusogenic molecules in both cell-to-cell fusion and perhaps virus-to-cell fusion...
mechanisms (Pertel, 2002). The actual mechanism of virus-induced membrane fusion or the associated viral molecules involved in mediating and/or regulating virus-to-cell or cell-to-cell fusion has yet to be fully understood; however, herpesviral fusion mechanisms are under intense investigation (Foster, Alvarez, and Kousoulas, 2003; Foster et al., 2004; Foster et al., 2003; Melancon, Foster, and Kousoulas, 2004). Recent studies on the KSHV-gB molecule has demonstrated the induction of signal transduction mechanisms upon binding of either virions or soluble gB molecules onto target cells; the signal transduction mechanisms seems to prepare the target cell for viral entry via morphological changes exhibited by the following: 1.) commencement of endocytic pathways, 2.) movement of particulate materials in the cytosol, and 3.) cytoskeletal rearrangements through the reorganization of actin (Akula et al., 2003; Akula et al., 2002; Akula et al., 2001b; Sharma-Walia et al., 2004; Wang et al., 2003).

**KSHV Latency**

One of the striking features of all herpesviruses is its ability to remain in a latent state for extended periods of time, KSHV latency could easily last decades before reactivation. KSHV is similar to other herpesviruses in which infections could present itself as either latent (nonproductive) or lytic (productive). A productive lytic infection of herpesviruses leads to large amounts of virions and cell death via lysis. Intuitively, lytic infections are not congruent with the events leading to cell transformation and eventual cancer; KSHV readily transforms cells leading to tumorigenesis in vivo (Ablashi et al., 2002; Boshoff and Weiss, 1998; Moore and Chang, 2003).

EBV latent infection and the concomitant expression of the latent viral genes have been shown to be essential for the development of EBV-related lymphoproliferative...
disorders (Griffin, 2000). Analogous to EBV, KSHV establishes a latent infection in the vast majority of tumor cells in Kaposi’s Sarcoma lesions, thus implying that KSHV latent infection plays an essential role in the development of Kaposi’s Sarcoma lesions (Moore and Chang, 2001). Similar to EBV, KSHV exhibits two distinct latent and lytic DNA replication and gene expression programs in infected cells (Ballestas, Chatis, and Kaye, 1999; Ragoczy and Miller, 2001). KSHV latent and lytic DNA replication is crucial and necessary for the long-term persistence of the virus; moreover, both latent and lytic gene expression programs are involved in the pathogenesis of KSHV-related lymphoproliferative disorders (Cesarman, 2002; Jenner and Boshoff, 2002).

KSHV expresses seven genes, which constitute the latent gene expression program; this set of latent genes have been shown to manifest both growth transforming and cell cycle-deregulating properties thus providing a conducive milieu for KS pathogenesis (Cesarman, 2002; Cotter and Robertson, 2002; Komatsu et al., 2002). The KSHV genes expressed in the latent program are the following: LANA-1 (ORF73), v-cyclin D (ORF72), v-FLICE (K13), kaposin A (K12), v-IRF-2 (K11.5), LANA-2 (K10.5) and LAMP (K15). LANA-1 is perhaps the most important latent gene expressed during the latent cycle, since it has been shown by several investigators to be the most active and serve a myriad of roles: 1.) binds transcriptional corepressors (SAP30, Sin3A, CIR), 2.) possesses a nuclear localization motif, 3.) binds KSHV TR DNA cooperatively and supports TR directed viral replication, 4.) binds host chromatin proteins, 5.) binds and inhibits p53, 6.) binds RB1, 7.) transcriptional activation through E2F, 8.) transcriptional activation of EBV latency promoters, 9.) binds HIV-1 Tat, and 10.) binds to KSHV-Rta, the KSHV major transactivator (Dourmishev et al., 2003; Lan et al., 2004).
LANA-1, v-cyclin D and v-FLICE (K13) are latent transcripts present and expressed from a differentially spliced polycistronic mRNA, in which their transcription is regulated via a common promoter (Cesarman et al., 1996; Dittmer et al., 1998; Grundhoff and Ganem, 2001; Sarid et al., 1999; Talbot et al., 1999). LANA-1 has been consistently shown to be the immunodominant latent antigen expressed in KS lesions (Dupin et al., 1999; Gao et al., 1996a; Gao et al., 1996b; Kedes et al., 1997; Kedes et al., 1996; Kellam et al., 1997; Rainbow et al., 1997). LANA-1 consists of 1,162 amino acid and is identified as protein doublets of 222- to 234-kDa in infected cell lines by Western blot analysis (Kedes et al., 1997; Rainbow et al., 1997). LANA-1 exhibits a punctuate nuclear distribution pattern in immunohistochemical experiments (Gao et al., 1997; Gao et al., 1996b; Kedes et al., 1996). LANA-1 has been shown to be a multifunctional protein able to autoactivate its own promoter (Jeong, Papin, and Dittmer, 2001; Renne et al., 2001). Viruses that establish latent infection must maintain their DNA in the host nucleus through many cellular generations; thus KSHV must replicate its latent circular genome before each cell division and then successfully segregate into progeny cells.

KSHV latently infected cells harbor several copies of an extrachromosomal circular KSHV DNA, also known as the KSHV episomes. In uninfected B lymphoblastoid cells, the expression of KSHV LANA-1 allows for the persistence of an episome containing the KSHV terminal repeat (TR) sequence (Ballestas, Chatis, and Kaye, 1999; Ballestas and Kaye, 2001); thus showing a necessary and sufficient role for LANA-1 in KSHV episome persistence in transfected cells Ballestas, 1999 #1325; Ballestas, 2001 #794}. Furthermore, LANA-1 has been shown to specifically bind KSHV TR DNA and allow for its replication (Cotter and Robertson, 1999; Cotter, Subramanian, and Robertson,
The gene expression profile of genes within a given cell determines the cell fate and is in a large part due to effects of chromatin remodeling and structure (Litt et al., 2001). In KSHV infected pleural effusion lymphoma cells, the majority of cells harbor KSHV in a latent state, in which most of the viral genes are silenced; the precise mechanisms of viral gene silencing are unknown (Renne et al., 1996b). LANA-1 has been shown to localize at the host heterochromatin region, an area where the majority of the genes are inactive or silenced (Szekely et al., 1999). The chromatin protein SUV39H1 methylates the histone H-3 protein thus allowing for the Heterochromatin protein 1 (HP-1) to bind to the methylated histone H-3 protein and expand the heterochromatin region (Aagaard et al., 1999; Bannister et al., 2001; Lachner et al., 2001). A recent study has shown that LANA-1 can specifically recruit SUV39H1 and HP-1 to the KSHV genome thus leading to formation of heterochromatin, which could in part explain the localization of the KSHV latent genome in the heterochromatin (Sakakibara et al., 2004).

KSHV has been shown to associate with mitotic chromosomes (Ballestas, Chatis, and Kaye, 1999; Krithivas et al., 2002; Piolot et al., 2001; Szekely et al., 1999); immunofluorescence experiments have shown that the concentration of LANA-1 at sites of KSHV DNA along mitotic chromosomes (Ballestas, Chatis, and Kaye, 1999; Cotter, Subramanian, and Robertson, 2001; Jones et al., 1998; Szekely et al., 1998). During latency, LANA-1 binds specifically to the TR region of DNA repeat sequences consisting of 801 base-pair units and containing an origin of replication (OriP) (Ballestas, Chatis,
and Kaye, 1999; Ballestas and Kaye, 2001; Garber et al., 2001). The tethering of the KSHV genome by LANA-1 is also done by its interaction with host chromatin proteins such as histone H1, MeCP, DEK, RING3 (Cotter, Subramanian, and Robertson, 2001; Krithivas et al., 2002; Mattsson et al., 2002). Unlike other KSHV promoters tested thus far, LANA promoter is not affected by tetradecanoyl phorbol acetate or viral lytic cycle functions. It is, however, subject to control by LANA itself and cellular regulatory factors, such as p53.

**Viral Gene Transcription and Expression**

Traditionally, transcription of herpesvirus genes is tightly regulated and is sequentially ordered in a cascade fashion (Knipe et al., 2001). The sequentially ordered cascade of transcription occurs via a simultaneously sequentially ordered viral protein cascade which modulates viral gene expression. Herpesviruses conscript the host RNA polymerase and other members of the host transcriptional apparatus in order to execute its viral transcriptional program; transcription of herpesviral DNA occurs in the nucleus and the viral proteins are synthesized in the cytoplasm (Knipe et al., 2001). Herpesviral genes are categorized into mutually exclusive latent and lytic profiles. This dichotomy of herpesviral gene transcription involves the latent phase which occurs while the viral genome is maintained as an episome and a lytic phase which takes place in a cascade fashion during productive (lytic) infection. Upon establishment of a latent infection (nonproductive) only a handful of genes are expressed. During the course of a productive (lytic) herpesviral infection, approximately eighty genes are expressed. Lytic infection of KSHV leads to cell lysis and therefore cell death, which is counterintuitive and inconsistent with the establishment of KSHV transformation of the infected cell. A
strikingly low level of KSHV infected PEL cultures undergo spontaneous lytic gene expression which could be readily detected in a stable milieu of PEL cultures that exhibit latent expression; the infrequent spontaneous KSHV-reactivation correlates well with the infrequent reactivation detected in KS clinical samples (Fakhari and Dittmer, 2002; Jenner et al., 2001; Paulose-Murphy et al., 2001; Sarid et al., 1998). The majority of infected cells in KS specimens exhibit latent KSHV gene expression, with a scant number of cells expressing lytic transcripts (Chan, Bloomer, and Chandran, 1998; Dupin et al., 1999; Katano et al., 2000; Lin, Dai, and Ricciardi, 1998; Orenstein et al., 1997; Parravicini et al., 2000; Staskus et al., 1997; Sun et al., 1999), thus implying that the low level of spontaneous lytic gene expression is not an artifact of tissue culture models.

Recently, models of de novo infection of cultured endothelial cells have also manifested a similar “hodge-podge” pattern of latent and lytic gene expression (Ciufò et al., 2001; Lagunoff et al., 2002; Moses et al., 1999). Both latent and lytic cycle replication are crucial for the long-term persistence of the virus, and gene products from both latent and lytic gene expression programs have been implicated in the pathogenesis of KSHV-associated disorders (Cesarman, 2002; Jenner and Boshoff, 2002).

Classification of the latent or lytic gene expression of individual KSHV ORFs would serve an immense role in the prediction of their potential contribution to the pathogenesis of the KSHV infection. The facilitation of cultured PEL cells latently infected with KSHV and inducing lytic reactivation with common laboratory chemicals (such as phorbol esters or sodium butyrate) have led to clear assignation of individual KSHV genes to either the latent or lytic gene expression. Typically, PEL cell lines (in which every cell in the PEL culture is infected with KSHV) carry an approximately 40 to
150 copies of KSHV DNA per cell genome (Drexler et al., 1998). Upon routine passage of the PELs, the virus is consistently maintained as a latent episome, with concomitant restriction of lytic viral gene expression and a paucity of virus production. Upon chemical induction of PELs, viral gene expression switches from the latent program to an ordered cascade of lytic gene expression, leading to viral replication, virion production, cell lysis, and viral release (Renne et al., 1996a; Renne et al., 1996b; Sarid et al., 1998; Zhong et al., 1996).

The expression pattern of each KSHV ORF was categorized into three classes of gene transcription: I, II, and III; the assignation of each ORF to its respective class was determined by the individual gene response to the addition of TPA to PEL cultures (Sarid et al., 1998). Class I transcripts are constitutively expressed and are not induced by phorbol esters. Class I gene transcripts correspond directly with the latent gene expression profile, which includes ORF73 (LANA-1), ORF72 (viral cyclin D [vCyc]) and K13 (fas-ligand IL-1 β-converting enzyme inhibitory protein [vFLIP]); these proteins are readily detected in KS samples further establishing the categorization within the latent phase of the KSHV transcriptional program (Davis et al., 1997; Dittmer et al., 1998). The three mentioned class I KSHV-ORFs have corresponding sequence homologs in HVS (Nicholas, Cameron, and Honess, 1992; Thome et al., 1997), and their respective expressed protein serve crucial roles in latency and cellular transformation (Dourmishev et al., 2003). ORF K13 (vFLIP) encodes a viral inhibitor of a cellular homologue of the Fas-mediated apoptosis (Thome et al., 1997). ORF 72 (vCyc) encodes a functional cyclin D homolog which could substitute for human cyclin D by phosphorylating the retinoblastoma tumor suppressor protein (Chang et al., 1996). ORF73 (LANA-1)
encodes LANA (Rainbow et al., 1997), a highly immunogenic protein that is highly expressed and is the basis for both immunofluorescence and Western assay-based serological tests (Gao et al., 1996a).

The second class of gene transcription, class II, includes mRNAs which are detected in variable abundance of high, moderate, or low in unstimulated cultures grown under standard growth conditions without phorbol esters, yet class II transcripts could be readily induced to higher levels of transcription by TPA (Sarid et al., 1998). Examples of class II mRNAs that are transcribed at moderate levels without TPA treatment include the following: cytokines v-IL-6 (ORF K2), v-MIP-II (ORF K4), and v-IRF (ORF K9). A true latent transcription is restricted in both PEL and KS lesions. However, a number of genes are expressed, generally at low transcription levels, in PEL without TPA treatment and are inducible with TPA (class II). The majority of class II genes includes typical herpesviral regulatory and viral DNA replicative genes, along with a majority of the viral homologs of cellular genes (Sarid et al., 1998). Interestingly, the class II category of transcription includes many unique KSHV genes (e.g., the viral cytokines and v-IRF), which lead many to believe that these transcripts serve essential roles in the manipulation of cellular pathways, regulation of viral infection and transformation of the host cell (Gao et al., 1997; Moore and Chang, 1998). Both class I and II genes are the least conserved among all herpesviruses, and these genes tend to cluster within the same region of the KSHV genome; this region containing regulatory genes are also referred to as “latency islands” (Knipe et al., 2001; Moore et al., 1996a; Moore and Chang, 2001).

The final class of gene transcription, class III, involves transcripts that could only be detected after induction with the phorbol esters (Sarid et al., 1998). Class III
transcripts include lytic genes which are transcribed during active infection and are necessary for efficient lytic-viral replication and virion particle production; examples of class III include the following transcripts: ORF 25 (major capsid protein), ORF 6 (DNA polymerase), and ORF 22 (glycoprotein H) (Sarid et al., 1998). The class III category consists of many genes which are highly conserved among all herpesviruses and possess functional roles in DNA replication and virion morphogenesis (Knipe et al., 2001; Sarid et al., 1998).

**Reactivation**

Typically in traditional herpes simplex infections (the prototype herpesvirus), a tegument protein localizes to the nucleus and activates the immediate early gene expression, which in turn commences the sequentially ordered cascade of viral gene expression characteristic of herpesviruses (Knipe et al., 2001). The two human gamma herpesviruses, EBV and KSHV, manifest similar patterns of the latent and lytic phases of infection, and both of these gammaherpesviruses require a reactivation mechanism in order to switch from the latency phase to the lytic phase. KSHV latency is critical in the establishment of a permanent infection both *in vitro* and *in vivo*, the latency phase permits the virus evasion of the host immune surveillance and to establish persistent infection (Moore and Chang, 2003). In addition, latent infection by either KSHV or EBV plays a major role in tumorigenesis (Cesarman, 2002; Chang and Moore, 1996). Due to KSHV’s characteristic establishment of latent infection in susceptible cells, viral reactivation must overcome a stringent regulation of viral gene expression, wherein viral gene expression is highly limited and tightly controlled. KSHV spontaneously reactivates in infected cells allowing for viral lytic replication. Both tetradecanoyl phorbol acetate...
(TPA) and sodium butyrate (NaB), have been shown to disrupt the latency of KSHV in BCBL-1 cells and induce lytic viral replication (Arvanitakis et al., 1996; Renne et al., 1996b; Yu et al., 1999). As mentioned in the previous section, five latent genes are expressed during latency: 1.) LANA-1, 2.) v-cyclin, 3.) v-FLIP, 4.) kaposin, and 5.) vIRF-2 (Burysek and Pitha, 2001; Dittmer et al., 1998; Muralidhar et al., 1998; Rainbow et al., 1997; Sadler et al., 1999; Saveliev, Zhu, and Yuan, 2002). The expression profile of these latent genes serve a crucial role in the maintenance of latency and cellular transformation, which at this point has not yet been fully understood. The current school of thought suggests that lytic reactivation of a low percentage of infected cells is necessary for KS development (Lukac, Kirshner, and Ganem, 1999; Martin and Osmond, 1999; Whitby et al., 1995). Accumulation of evidence over the years clearly suggests a role for reactivation and/or lytic replication in the pathogenesis and induction of KS tumors: 1.) linkage between the humoral immune response to KSHV infection and KS tumor progression (Whitby et al., 1995); 2.) augmentation of KSHV viral load in patients correlates with progression from asymptomatic phase to KS (Ambroziak et al., 1995); 3.) patients dually infected with both KSHV and HIV when treated with ganciclovir, a drug that effectively targets KSHV lytic replication, leads to a decreased incidence of KS development (Martin and Osmond, 1999), and 4.) both KS tumor cells (typically manifest a latent KSHV infection) and KSHV infected spindle cells (also typically manifest a latent KSHV infection); yet, KS tumor cells and KSHV infected spindle cells consistently exhibits a certain, albeit low, percentage of latent cells undergoing spontaneous reactivation with concomitant lytic replication (Reed et al., 1998; Staskus et al., 1999; Staskus et al., 1997; Zhong et al., 1996). Viral lytic reactivation and DNA replication is
considered crucial in KSHV spread from the lymphoid reservoir, B-cells, to endothelial cells thus leading to the KS spindle cell formation (Offermann, 1999). Hence the reactivation mechanisms regulating the latent to lytic replication switch in KSHV infected cells serves as an essential cog in the development of KS and the KSHV-related lymphoproliferative disorders.

**Regulation of Transcriptional Activation**

Upon phorbol ester induction of KSHV latently infected cells, the sequentially ordered cascade of the KSHV lytic lifecycle commences with rapidity due to the functional role of the KSHV-regulator of transcription activator (Rta). Although the majority of KSHV genes have been screened, the KSHV-Rta has been the only protein when overexpressed sufficient for viral reactivation (Gradoville et al., 2000; Lukac, Kirshner, and Ganem, 1999; Lukac et al., 1998; Sun et al., 1998). KSHV-Rta was initially identified based on positional analogies and sequence homology with EBV and HVS (Sun et al., 1998). The KSHV-Rta is one of the earliest immediate-early transcripts induced upon viral reactivation (Sarid et al., 1998; Sun et al., 1999; Zhu, Cusano, and Yuan, 1999). The major ORF50 transcript is a tricistronic mRNA, which also encodes the genes ORF-K8 (K-bZIP/RAP) and ORF-K8.1 (K8.1 glycoprotein) (Gruffat et al., 1999; Lin et al., 1999; Lukac et al., 1998; Seaman et al., 1999; Sun et al., 1998; Zhu, Cusano, and Yuan, 1999); alternative splicing mechanisms of the neighboring exons of this tricistron also leads to the expression of two minor tricistronic transcripts (Zhu, Cusano, and Yuan, 1999). The K-bZIP or RAP (ORF-K8) transcript is also produced with immediate-early kinetics independently of the upstream ORF50 gene but its role in reactivation has not been fully determined (Saveliev, Zhu, and Yuan, 2002). In clinical
samples, the Rta is detected corresponding with the lytic pattern in KS lesions (Katano et al., 2001; Sun et al., 1999). Rta, a 691 amino acid protein, has been shown to be highly posttranslationally modified and extensively phosphorylated (Lukac, Kirshner, and Ganem, 1999; Lukac et al., 1998). Numerous studies have shown via transfections experiments that Rta is capable of transactivating KSHV promoters of genes that are typically expressed upon a productive lytic infection (Chang et al., 2002; Chen et al., 2000; Jeong, Papin, and Dittmer, 2001; Lukac et al., 2001; Lukac, Kirshner, and Ganem, 1999; Lukac et al., 1998; Song et al., 2001; Wang et al., 2001b; Zhang, Chiu, and Lin, 1998). Rta possesses a transcriptional activation domain located at its carboxyl terminus domain which exhibits a similar structure conserved among several eukaryotic transcriptional activation domains (Lukac et al., 1998); deletion of this transcriptional activation domain generates an Rta-specific dominant negative inhibitor of transactivation Rta which upon transfection into BCBL-1 effectively suppresses spontaneous lytic reactivation from latency along with suppression of viral replication induced by the following chemical agents: TPA, sodium butyrate, and ionomycin (Lukac, Kirshner, and Ganem, 1999). Rta binds directly to various viral promoters with specific sequences (Chang et al., 2002; Deng et al., 2002b; Liang et al., 2002; Lukac et al., 2001; Song et al., 2002; Song et al., 2001). The majority of the targets of Rta transactivation are considered essential for lytic replication, Rta has been shown to transactivate the following genes which are considered essential for the virus to subvert normal regulatory cell growth mechanism: kaposin, vIL-6, vMIP-I, vIRF-1, vGPCR, and K1 (Bowser, DeWire, and Damania, 2002; Chen et al., 2000; Curreli et al., 2002; Deng et al., 2002b; Ueda et al., 2002; Wang et al., 2001b). The significance of Rta in the dysregulation of
cellular growth pathways is shown by Rta-mediated activation of cellular IL-6 (Deng et al., 2002a), the ability of Rta to block p53-mediated apoptosis via competitive binding to CBP (Gwack et al., 2001). Concordant with the orchestration of the KSHV lytic cycle by Rta with concomitant manifestation of pathogenic progression, a clinical study has shown the Rta promoter is repressed by methylation and upon demethylation of the promoter latent KSHV genome is induced and begin lytic replication (Chen et al., 2001).

**Viral DNA Replication**

As a gamma-herpesvirus, KSHV possesses both latent and lytic replication cycles (Miller et al., 1997; Renne et al., 1996a). However, latency expresses only a minimal number of viral genes, and no infectious virus is produced during this phase. In KSHV latently infected cells, multiple copies of the viral genome are harbored and maintained as extrachromosomal episomes and latent KSHV DNA replication is synchronized with host cell division (Ballestas, Chatis, and Kaye, 1999). The terminal repeat (TR) sequence in the KSHV genome is necessary and sufficient for persistence of the viral episome and potentially serves as the origin of latent plasmid replication (ori-P) (Ballestas and Kaye, 2001). LANA-1 binds to cis-acting region within the KSHV TR DNA and acts in trans on the ori-P to mediate episome persistence (Ballestas and Kaye, 2001). Upon KSHV reactivation with the concomitant disruption of latency, the virus switches to a lytic life cycle which entails the facilitation of its lytic encoded replication proteins (Miller et al., 1997; Renne et al., 1996a). During the viral lytic component of the lifecycle, KSHV expresses the majority of its genes, and viral DNA is amplified by a viral encoded replication apparatus different from the latent viral DNA replication (Gradoville et al., 2000; Wu et al., 2001).
In herpesviruses, lytic DNA replication is different in two regards from the latent DNA replication. Firstly, upon lytic DNA replication viral DNA is amplified from a range of 100 to a 1,000-fold via a rolling circle mechanism, which produces viral progeny in concatemeric molecules also referred to as “head-to-tail” concatemers (Knipe et al., 2001). This rolling circle replication which occurs during the lytic phase of the viral lifecycle is in stark contrast to latent DNA replication which occurs simultaneous with the host cell division with the subsequent effect of maintaining a stable and low number of viral episomes. Secondly, lytic herpesviral DNA replication employs its own DNA replication machinery replete with its own DNA polymerase and associated viral components of the replication apparatus (Knipe et al., 2001). The herpesviral-encoded lytic replication apparatus is another distinction from the herpesviral latent DNA replication which requires the host cellular DNA polymerase along with its accessory proteins.

The herpesviral lytic DNA replication is initiated from an origin (ori-Lyt) and requires many viral gene products. The origin region or ori-Lyt is bound by a virus-encoded origin-binding protein (OBP) that recruits the core replication machinery. A number of herpesvirus lytic origins have been identified and characterized (Anders et al., 1992; Anders and Punturieri, 1991; AuCoin et al., 2002; Hammerschmidt and Sugden, 1988; Pari et al., 2001; Stow, 1982; Stow and Davison, 1986). These studies indicate several common features that have been identified within the herpesviral lytic origins, such as, AT-rich regions that are presumably sites where DNA is melted or unwound, numerous transcription factor-binding sites, and promoter enhancer elements, that are associated with active transcription or assembly of the transcription machinery. In the
KSHV genome, two copies of the lytic DNA replication origin [ori-Lyt (L) and ori-Lyt (R)] have been identified (AuCoin et al., 2002; Lin et al., 2003). The first ori-Lyt is located in the KSHV genome between K4.2 and K5 and the second ori-Lyt is located between K12 and open reading frame 71 (ORF71). KSHV lytic origins closely resemble the lytic origin of a related herpesvirus, rhesus macaque rhadinovirus (RRV) (Pari et al., 2001). Both RRV and KSHV lytic origins have GC-rich regions proximal to an AT-rich region. Initial mapping studies indicated that replication was dependent on the presence of the AT-rich region and a portion of GC-rich region (AuCoin et al., 2002; Pari et al., 2001).

The lytic herpesviral replication apparatus involving all of its necessary viral trans-acting factors required for origin dependent DNA replication has been described for the Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), and herpes simplex virus type 1 (HSV-1) using a transient co-transfection replication assay (AuCoin et al., 2004; Fixman, Hayward, and Hayward, 1995; Pari et al., 2001; Sarisky and Hayward, 1996; Wang, Zhang, and Montalvo, 1998; Wu et al., 1988). A common theme to the herpesviral replication apparatus, which spans the three herpesviral subfamilies, is the requirement of six core replication proteins for ori-Lyt-dependent DNA replication: DNA polymerase, processivity factor, helicase, primase, primase-associated factor, and a ssDNA binding protein (Knipe et al., 2001). The KSHV genome encodes a set of six genes which have varying levels of homology to their respective core replication gene counterparts in EBV, HSV, and HCMV; the six KSHV core replication proteins are listed as followed: ORF9 (POL; DNA Polymerase), ORF59 (PPF; polymerase processivity factor or DNA replication protein), ORF6 (SSB; single-stranded DNA binding protein),
ORF56 (PRI; component of DNA helicase-primase complex), ORF40/41 (PAF; polymerase accessory factor), and ORF44 (HEL; component helicase-primase complex).

Importantly, each herpesvirus encodes an initiator protein or an origin binding protein, which allows the viral replication proteins to assemble and dock onto the ori-Lyt. The Zta protein has been shown to be essential for EBV origin-dependent DNA replication and serves to activate transcription as well as play a direct role in DNA replication (Chang et al., 1990; Sarisky and Hayward, 1996). In terms of KSHV, Rta serves as the major transactivator of gene expression and its presence has been shown to be sufficient for KSHV viral reactivation. However, another protein displaying delayed early kinetics, K-bZIP (K8, RAP), is postulated to be the initiator protein and may have a direct role in the initiation of DNA replication (Lin et al., 2003).

KSHV Glycoproteins and Their Putative Functions

The Kaposi’s sarcoma-associated herpesvirus genome encodes several glycoproteins, some of which possess significant homology to glycoproteins of other herpesviruses. These include glycoprotein B (gB[ORF8]), gH (ORF22), gM (ORF39), gL (ORF47) (Neipel, Albrecht, and Fleckenstein, 1997; Russo et al., 1996), and gN (ORF53) (Koyano et al., 2003). In addition, the K8.1 and vOX2 (K14) glycoproteins are unique to KSHV, with no counterparts in other herpesviruses (Chandran et al., 1998; Chung et al., 2002; Neipel, Albrecht, and Fleckenstein, 1997; Schulz, 1998).

Glycoproteins serve various functions at key points along the virus replicative cycle which include the following: virus attachment, penetration, cell-to-cell spread, egress, and virus-induced cell fusion. This section will review the features of these glycoproteins and describe the putative functions associated with each glycoprotein. Description of the
K8.1 glycoprotein is not included in this section, since an extensive discussion of K8.1 is located in the next chapter.

**Glycoprotein B (gB)**

Glycoprotein B (gB) is conserved across all subfamilies of herpesviruses. gB exists on the surface of infected cells or on virion envelopes as a homodimeric membrane protein that is N-glycosylated at multiple sites (Claesson-Welsh and Spear, 1986; Claesson-Welsh and Spear, 1987; Horsburgh et al., 1999; Ligas and Johnson, 1988; Roop, Hutchinson, and Johnson, 1993; Spear et al., 2003). Herpesviral gB displays the highest degree of conserved homology among the glycoproteins in the *Herpesviridae* family; moreover, the carboxy-tail domain of gB exhibits the highest degree of conserved homology within the gB molecule (Goltz et al., 1994; Pereira, 1994; Ross et al., 1989). A wealth of evidence indicates that gB plays important roles in membrane fusion phenomena during virus entry and virus-induced cell fusion: HSV-1 mutant viruses lacking gB are not able to enter into cells (Cai et al., 1987) due to a post-attachment defect that can be resolved by polyethylene glycol mediated fusion of viral envelopes with cellular membranes (Cai, Gu, and Person, 1988). Point mutagenesis in which single amino acid substitutions and/or truncations of the carboxyl terminus of gB cause extensive virus-induced cell fusion (Baghian et al., 1993; Bzik et al., 1984; Cai et al., 1988; Haan, Lee, and Longnecker, 2001). Transient transfection experiments showed that the transient co-expression of HSV-1 gB along with gD, gH and gL causes cell-to-cell fusion, which is substantially increased by carboxyl terminal truncations of gB (Highlander et al., 1991; Kousoulas, Person, and Holland, 1978; Pogue-Geile et al., 1984). Similarly, transient transfection experiments using KSHV-gB along with gH and
gL were sufficient to cause cell-to-cell fusion; however, upon deletion of the final 59 a.a. of the KSHV-gB carboxyl tail (termed gB-Mut) cell-to-cell fusion was significantly enhanced. These results suggest a direct role for gB in membrane fusion and suggest that perturbations of the carboxyl terminal domains of gB facilitate gB-mediated cell-to-cell fusion.

The KSHV-gB molecule, similar to other herpesviral glycoproteins categorized as type I integral membrane proteins, consists of four domains: 1.) signal peptide domain, 2.) ectodomain (the domain of the molecule which is exposed towards the outside of the cell), 3.) transmembrane domain, and 4.) carboxyl-tail domain (domain which is hydrophilic and located within the cytosol). The first 23 amino acids (a.a.) of KSHV-gB constitute the putative signal peptide and amino acids 733 through 752 constitute the transmembrane domain of gB, thus the KSHV-gB molecule can be categorized into the ectodomain (703 a.a.) and the carboxyl terminal tail (93a.a.) (Pertel, 2002; Pertel, Spear, and Longnecker, 1998; Wang et al., 2003). The carboxyl tail of KSHV-gB manifests characteristics similar to the cytoplasmic terminal domain of HSV1-gB, in which the amino acid residues in this region are hydrophilic and positively charged (Pellett et al., 1985; Pertel, Spear, and Longnecker, 1998). The KSHV-ORF8 encodes the gB gene (Russo et al., 1996). Based on its primary structure the predicted molecular weight of KSHV-gB would be expected to be 91.3 kDa; however, studies have shown that a fully processed gB could be detected well over 100 kDa (Akula et al., 2001a; Baghian et al., 2000; Pertel, Spear, and Longnecker, 1998). KSHV-gB possesses thirteen possible N-linked glycosylation sites (Asn-X-Thr/Ser motif whereby the X is any amino acid except proline (Bause, 1983; Gavel and von Heijne, 1990; Marshall, 1972). Glycosylation
inhibition experiments using both N-glycosidase F (Endo F), endoglycosidase H (Endo H) (Baghian et al., 2000; Pertel, Spear, and Longnecker, 1998) and tunicamycin (an antibiotic which is an inhibitor of N-linked glycosylation) (Baghian et al., 2000) have clearly shown that the KSHV-gB contains predominantly N-linked carbohydrates.

In virion preparations produced from BCBL-1 cells, it has been shown that the virion envelope-associated gB is present on the surface of both infected cells and the virion envelope (Akula et al., 2001a; Baghian et al., 2000). Among the several glycoproteins on the envelope of the KSHV particle, virion envelope-associated gB has been shown to exhibit strong affinity for heparin as demonstrated by experiments in which virion envelope-associated gB binds to heparan-agarose beads, and binding of a biotinylated peptide of a gB-heparan binding domain (gB-HBD peptide consisted of the following 108-117 gB-a.a. sequence: HIFKVRYYRK binds in a dose-dependent manner to BSA-Heparan coated 96 well plates(Akula et al., 2001a).

The role of gB in viral attachment and penetration is clearly shown as rabbit polyclonal anti-gB antibodies block KSHV infectivity in human foreskin fibroblast cells (HFF) in a dose-dependent manner; the rabbit polyclonal anti-gB antibodies were raised in New Zealand white male rabbits which were immunized with purified GST-gB fusion protein (Akula et al., 2001a). The role of KSHV-gB in viral entry was further established in a study which demonstrated that the integrin α3β1 molecule is one of the cellular receptors utilized by KSHV for viral entry into susceptible cells; KSHV was the first herpesvirus shown to utilize integrin as a cellular receptor for target cells (Akula et al., 2002; Nemerow and Cheresh, 2002). The RGD (Arg-Gly-Asp) amino acids constitute a motif which is necessary for a myriad of cellular ligands that bind to host cell surface
integrin molecules (Plow et al., 2000). Immediately behind the KSHV-gB signal peptide, the gB molecule contains an RGD motif (a.a. 27-29) which binds specifically to the integrin α3β1 receptor (CD49c/29) (Akula et al., 2002; Wang et al., 2003). Interestingly, the gB/integrin interaction is not a common feature of herpesvirus gBs; there has been no other gB in the entire Herpesviridae family that possesses a similar conserved RGD motif. In a dose-dependent manner, peptides with the RGD motif efficiently blocked KSHV infection in a range from 40-80% and antibodies raised against a peptide with the gB-RGD sequence also blocked KSHV infection up to 60% in a dose-dependent manner (Akula et al., 2002). Integrin α3β1 is broadly expressed and has been detected on all cells susceptible to infection by HHV-8, including human foreskin fibroblasts and B, epithelial, endothelial, and 293 cells (Akula et al., 2002; Plow et al., 2000; Wu and Dedhar, 2001). Both soluble integrin α3β1 protein and anti-integrin antibodies against efficiently blocked KSHV infectivity up to 80% and 50%, respectively (Akula et al., 2002). Furthermore, the inability of these molecules to completely neutralize viral infectivity leads to implications of potentially other cellular receptors utilized by KSHV for entry. Interestingly at similar concentrations used for the above mentioned virus neutralization experiments, RGD peptides, RGD-gB antibodies, anti-integrin antibodies and soluble integrin α3β1 proteins could not inhibit KSHV binding to HFF cells yet soluble heparin almost completely blocks viral attachment, thus suggesting a post-attachment role for the gB/integrin interaction, in which gB binding to the integrin receptor is able to facilitate viral entry into susceptible cells (Akula et al., 2002).

Envelope-associated KSHV-gB clearly binds to both heparan sulfate receptors (HS) and integrin α3β1 which are located on the surface of target cells, thus two different
motifs (HBD and RGD) of KSHV-gB mediates KSHV binding via HS and viral entry via integrin $\alpha_3\beta_1$ cellular receptor, respectively (Akula et al., 2001a; Akula et al., 2002; Akula et al., 2001b). Interestingly, soluble gB proteins upon binding to target HFF cells induce morphological changes, e.g., cell rounding and detachment, yet these cells remained viable with no noticeable cellular death (Akula et al., 2003). These results are consistent with the morphological changes noticed by integrin-mediated clumping of integrin molecules with concomitant rearrangement of the actin cytoskeleton (van der Flier and Sonnenberg, 2001).

**Glycoprotein H (gH) and Glycoprotein L (gL)**

The products of the KSHV-ORF22 and KSHV-ORF47, gH and gL respectively form heterodimers which are components of the virion envelope (Naranatt, Akula, and Chandran, 2002). The virion envelope embedded glycoprotein H (gH) has been highly conserved among all members of the herpesvirus family, gH has been shown to be essential for virus penetration and cell-to-cell spread (Babic et al., 1996; Duus, Hatfield, and Grose, 1995; Forghani, Ni, and Grose, 1994; Fuller, Santos, and Spear, 1989; Haddad and Hutt-Fletcher, 1989; Hutchinson et al., 1992; Khattar et al., 1996; Klupp et al., 1997; Li, Turk, and Hutt-Fletcher, 1995; Lomonte et al., 1997; Mukai et al., 1997; Strnad et al., 1982; Wu, Reed, and Lee, 2001; Wu et al., 1999). Expression of gH in the absence of gL leads to a protein that is incorrectly folded and processed. Heterodimeric complex formation between gH and gL is a common theme among herpesviruses: Herpes simplex virus-type1 (HSV-1) (Hutchinson et al., 1992), varicella zoster virus (VZV) (Forghani, Ni, and Grose, 1994), human cytomegalovirus(HCMV) (Kaye, Gompels, and Minson, 1992), human herpesvirus 6 (HHV-6) (Liu et al., 1993), HHV-7 (Mukai et al., 1997),
Epstein-Barr virus (EBV) (Yaswen et al., 1993). However, a heterocomplex is formed in EBV, the gH, gL, and gp42 complex is required for the infectivity of B cells, yet gp42 is not required for the infectivity of epithelial cells (Li, Turk, and Hutt-Fletcher, 1995). KSHV gL is required for the proper processing and transport of KSHV gH to the infected cell membranes. Anti-gH and anti-gL rabbit antibodies neutralized KSHV infectivity without inhibiting the binding of virus to the target cells, suggesting that gH and gL play an important role in the post-binding step of KSHV infection. KSHV gH and gL expression can be readily detected during lytic replication upon the addition of TPA to BCBL-1 cells (Naranatt, Akula, and Chandran, 2002). KSHV-gL has been shown to be independently expressed on cell surfaces. Independent expression of gH without gL causes gH misfolding and aggregation of gH in the ER; however, coexpression of KSHV-gH with KSHV-gL facilitates the correct processing of gH and mediates transport through the golgi apparatus with subsequent cell surface expression (Naranatt, Akula, and Chandran, 2002). Antibodies to either gH and gL do not inhibit KSHV binding, yet anti-gH and anti-gL antibodies do neutralize KSHV infectivity at a post-attachment step of HHV-8 infection (Naranatt, Akula, and Chandran, 2002).

**Glycoprotein M (gM) and Glycoprotein N (gN)**

The glycoproteins M and N, (gM and gN), have been conserved throughout the entire family, Herpesviridae. The products of the KSHV-ORF39 and KSHV-ORF53 are gM and gN, respectively. Both KSHV-gM and gN are N-glycosylated and form heterodimers as shown by immunoprecipitation experiments. The heterodimer formation between gM and gN is consistent with heteroduplex formation in other herpesviruses as well (Jons, Dijkstra, and Mettenleiter, 1998; Lake, Molesworth, and Hutt-Fletcher, 1998;
Mach et al., 2000; Rudolph et al., 2002; Tischer et al., 2002; Wu, Zhu, and Letchworth, 1998). The glycosylated forms of gM are observed at 46 and 80 kDa and upon the addition of tunicamycin, an inhibitor of N-glycosylation, the molecular sizes are reduced to 39 and 71 kDa. The glycosylated form of gN is approximately 26 kDa and upon tunicamycin treatment the unglycosylated form is approximately 18 kDa. gN was shown to be required for proper post-translational modification and transport of gM to both the cell surface (Koyano et al., 2003). In addition, gM was shown to be present on the surface of the virion envelope, since an anti-peptide antibody directed against gM reacted with double-purified sucrose gradient centrifugations (Koyano et al., 2003). The KSHV-gM and -gN heterocomplex was shown to inhibit cell fusion in an in vitro HSV-1 and Mo-MuLV (Molony murine leukemia virus) cell fusion assay (Koyano et al., 2003).

**Glycoprotein OX2 (vOX2)**

Viral genomic analysis of KSHV suggests that ORFK14 possesses a significant level of homology with cellular OX2, currently designated as viral OX2 (vOX2). Cellular OX2 belongs to a group of leukocyte glycoproteins which are expressed on the surface of a myriad of cells: activated T cells, B cells, follicular dendritic cells, neurons, and vascular endothelial cells (Wright et al., 2003; Wright et al., 2000). The respective receptor for CD200 is CD200R which has been primarily found mainly on cells of myeloid origin (Wright et al., 2003; Wright et al., 2000). Recently, a study indicated that the CD200/CD200R interaction could enact its immunosuppressive effect via T-cell involvement, since the receptor, CD200R, is also present on the surface of certain T-cells (Wright et al., 2003). CD200 possesses a small carboxy-tail without noticeable signal transduction motifs; however, the CD200R possesses a large cytoplasmic tail with
tyrosine-based signaling motifs, which potentially could be used to deliver its restrictive immunological effect on myeloid cells (Wright et al., 2003). The KSHV ORFK14 encodes the vOX2 specifying 271 amino acids, which is analogous to both the cellular OX2 (278 amino acids) and *rhesus rhadinovirus* RRV ORF-R14 (253 amino acids), which is a homologue of KSHV vOX2 (Alexander et al., 2000; Searles et al., 1999). Similar to both cellular OX2 and RRV R14 homologues, the predicted KSHV vOX2 contains a typical signal peptide sequence at its amino-terminal region and contains five potential N-glycosylation sites in its extracellular domain. ORFK14 (vOX-2) exhibits a low 40% DNA sequence identity to cellular OX-2. vOX-2 binds to the receptor CD200R with almost identical affinity and kinetics as the cellular OX-2 (Chung et al., 2002). The c-OX2-OX2R interaction delivers a restrictive signal to macrophages and thus limits macrophage activation. Subsequently, blocking of this interaction with anti-OX2R antibody exacerbates tissue damage in inflammatory sites (Wright et al., 2000). A recent study has shown that the KSHV ORF-K14, vOX-2, can inhibit TNF-α secretion from activated macrophages which is mediated by cell surface interaction of CD200R on the surface of macrophages with vOX-2 expressed on the surface of KSHV-infected cells undergoing viral lytic replication (Foster-Cuevas et al., 2004). vOX2 encodes a glycosylated protein with an apparent molecular mass of 55 kDa, is expressed on the surface of KSHV-infected cells during viral lytic replication, and specifically recognizes myeloid-lineage cells (Chung et al., 2002). The fact that viral homologs to CD200 [Betaherpesvirinae (HHV-6 (Gompels et al., 1995) and HHV-7 (Muralidhar et al., 2000); Gammaherpesvirinae (rhesus macaque rhadinovirus (Desrosiers et al., 1997; Searles et al., 1999) and KSHV (Chang et al., 1994); along with numerous viruses within the Pox
family (Cameron et al., 1999; Lee, Essani, and Smith, 2001; Willer, McFadden, and Evans, 1999) span large taxonomical gaps, suggests a common viral immunoevasion strategy which negatively restricts activated macrophages, thus potentially allowing a milieu conducive for KS sarcomagenesis (Chung et al., 2002; Foster-Cuevas et al., 2004).

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CHAPTER II

KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS GLYCOPROTEIN K8.1 IS DISPENSABLE FOR VIRUS ENTRY

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV), also referred to as human herpesvirus 8 (HHV-8) is a member of the γ2 herpesvirus family (genus Rhadinovirus) (Neipel, Albrecht, and Fleckenstein, 1997; Russo et al., 1996). KSHV is etiologically associated with Kaposi’s sarcoma (KS), primary effusion/body cavity-based lymphoma (PEL/BCBL) and multicentric Castleman’s disease (Antman and Chang, 2000; Ganem, 1998; Schulz, Chang, and Moore, 1998; Schulz, Sheldon, and Greensill, 2002). Recently, it was suggested that KSHV may have a role in the development of primary pulmonary hypertension (Cool et al., 2003). KSHV can infect a variety of human cell types, including B, T, endothelial, epithelial, fibroblast and keratinocyte cells, and nonhuman cell types, including owl monkey kidney and baby hamster kidney fibroblast cells (Cerimele et al., 2001; Ciufò et al., 2001; Flore et al., 1998; Foreman et al., 1997; Gao, Deng, and Zhou, 2003; Kliche et al., 1998; Mesri et al., 1996; Moore et al., 1996; Moses et al., 1999; Panyutich, Said, and Miles, 1998; Renne et al., 1998; Vieira et al., 2001; Zhou et al., 2002).

Generally, all herpesviruses initiate infection via direct binding onto various receptors on cell surfaces mediated by several viral glycoproteins embedded into viral envelopes. Viral glycoproteins play important roles in virus attachment onto susceptible
cells, fusion of the viral envelope with either cellular or endosomal membranes, and
virion morphogenesis and egress [reviewed in: (Kieff and Rickinson, 2001; Mocarski and
Courcelle, 2001; Roizman and Knipe, 2001)]. Herpes simplex virus (HSV), human
cytomegalovirus (HCMV), and Epstein Barr Virus (EBV) have been shown to enter into
cells via either pH-independent or pH-dependent pathways depending on the cell type
(Bodaghi et al., 1999; Compton, Nepomuceno, and Nowlin, 1992; Miller and Hutt-
Fletcher, 1992; Nemerow and Cooper, 1984; Nicola, McEvoy, and Straus, 2003;
Roizman and Knipe, 2001; Spear, 1993). KSHV has been shown to enter into certain
cells (Human foreskin fibroblast cells and B cells) via endocytosis (Akula et al., 2003;
Akula et al., 2001b). Regardless of the mode of virus entry, release of capsids into the
cytoplasm is thought to involve fusion of the viral envelope with either plasma or
endosomal membranes.

KSHV codes for a number of glycoproteins, some of which have significant
homology to glycoproteins of other herpesviruses. These include gB(ORF 8), gH (ORF
22), gM (ORF 39), gL (ORF 47) (Neipel, Albrecht, and Fleckenstein, 1997; Russo et al.,
1996), and gN (ORF 53) (Koyano et al., 2003). In addition, K1, K8.1 and vOX2 (K14)
glycoproteins are unique to KSHV with no counterparts in other herpesviruses (Chandran
et al., 1998; Chung et al., 2002; Neipel, Albrecht, and Fleckenstein, 1997; Schulz, Chang,
and Moore, 1998). KSHV glycoproteins gB and K8.1A mediate initial binding of virions
onto glycosaminoglycans, e.g. heparan sulfate on cell surfaces (Akula et al., 2001a;
Akula et al., 2001b; Birkmann et al., 2001; Wang et al., 2001). In agreement with the
strong binding of purified K8.1A to heparan sulfate moieties on cell surfaces, initial
studies showed that a soluble form of K8.1A inhibited KSHV attachment onto cells
(Wang et al., 2001). However, a later report indicated that a similar soluble form of K8.1A did not block KSHV infectivity (Birkmann et al., 2001). In addition, gB binds to integrins, such as α3β1 membrane receptors through a RGD motif, suggesting that integrins function as cellular receptors for KSHV entry (Akula et al., 2002; Naranatt et al., 2003). However, soluble integrins or RGD-containing peptides failed to inhibit virus entry into 293 cells (Inoue et al., 2003).

There are two ORFs originating from the K8.1 gene via spliced transcripts, K8.1A and K8.1B. The K8.1A cDNA encodes a 228 amino-acid (aa) protein containing a signal sequence, transmembrane domain and four N-glycosylation sites. The K8.1B cDNA encodes a 167 aa glycoprotein sharing similar amino and carboxy termini with K8.1A but contains an in-frame deletion (Chandran et al., 1998; Raab et al., 1998). K8.1A is the predominant form detected within infected cells and the virion envelopes (Zhu, Puri, and Chandran, 1999). The K8.1 gene has attracted significant interest due to the fact that it is positionally co-linear to the EBV major glycoprotein gp350/220 (Gong and Kieff, 1990), gp150 of murine gammaherpesvirus 68 (MHV 68) (Stewart et al., 1996), herpes virus saimiri (HVS) ORF 51 gene (Albrecht et al., 1992) and the BOEFD1 gene of bovine herpesvirus-4 (BHV-4) (Neipel, Albrecht, and Fleckenstein, 1997; Russo et al., 1996). EBV gp350/220 has been shown to be involved in the binding of the virus to the target cells via the CD21 receptor on B cells (Fingeroth et al., 1984; Nemerow et al., 1989; Nemerow et al., 1987; Nemerow et al., 1985; Tanner et al., 1987); however, gp350/220 is not required for virus entry into fibroblasts (Janz et al., 2000).

Recently, the KSHV genome was cloned into a bacterial artificial chromosome (BAC) and was shown to produce infectious virus (Zhou et al., 2002). To resolve whether
K8.1A functions in virus infectivity, we utilized the recombinant KSHV BAC36 as the initial template to construct a KSHV K8.1-null virus to address the role of K8.1 glycoproteins in the KSHV lifecycle. Our data indicate that both K8.1 glycoproteins are dispensable for virus entry.

**MATERIALS AND METHODS**

**Cells and Viruses**

293 cells were grown in Dulbecco’s modified Eagle medium (GIBCO-BRL; Grand Island, N.Y.) supplemented with 2 mM glutamine, 10% fetal calf serum (FBS) and antibiotics. The KSHV BAC36 virus contains the green fluorescent protein (GFP) gene cassette under the human cytomegalovirus immediate early promoter (HCMV-IE), constitutively expressing the GFP gene, inserted between KSHV ORF18 and ORF 19 (Zhou et al., 2002).

**Immunofluorescence Assay**

Detection of K8.1A was monitored by indirect immunofluorescence using FITC-conjugated goat anti-mouse IgG, which detected expression of the K8.1A plasmid in transiently transfected 293 cells. Transfected cells were incubated for 48 hours at 37°C, and then subsequently fixed with cold methanol for 20 minutes. Monolayers were blocked for 1 hour with 10% normal goat serum in PBS followed by 1 hour incubation with the primary monoclonal antibody (65), (19B4) directed against the K8.1 proteins, at a dilution of 1:500. Cells were then rinsed three times with PBS and incubated for 1 hour with the secondary antibody, a fluorescein-conjugated goat anti-mouse antibody (ICN Pharmaceuticals, Inc., Aurora, OH), diluted at 1:50. Cells were then washed 5 times with PBS and viewed via a fluorescence microscope.
Immunoblot Analysis

Cell lysates of K8.1A transfected 293 cells or induced BCBL-1 cells were boiled in loading buffer for 5 min and the proteins were separated by SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membranes, blocked with BLOTTO (5% nonfat milk in 0.01 M PBS/0.05% Tween 20), and then reacted for 1 hour with a primary antibody (K8.1A monoclonal; 19B4) at a dilution of 1:1000. A horse-radish peroxidase (HRP)-conjugated secondary antiserum at a dilution of 1:10,000 in PBS containing 10% goat serum was used. The reaction was visualized using the Western-blotting chemiluminescence detection reagents (Pierce Inc.; Rockford, IL).

Construction of a KSHV-Mutant with Deletion of the K8.1 Gene (BAC36ΔK8.1)

Mutagenesis of BAC36 DNA was accomplished in *Escherichia coli* using the λ gam, *recE recT* (GET) recombination system (Narayanan et al., 1999; Orford et al., 2000). Electrocompetent DH10B *E. coli* cells harboring BAC36 were transformed with the plasmid pGETrec, which contains the genes encoding *recE, recT* and bacteriophage λ *gam*, grown on plates containing chloramphenicol (12.5µg/ml) and ampicillin (100µg/ml). Individual colonies were picked and grown overnight in Luria-Bertani (LB) medium containing chloramphenicol and ampicillin. The next day, the culture was inoculated into 250ml of LB containing chloramphenicol and ampicillin until a 0.4 optical density was reached at 600nm. Addition of L-arabinose to a final concentration of 0.2% (w/v) and further incubation of 40 minutes induced the expression of the *recE, recT* and λ *gam* genes from the plasmid pGETrec. The cells were then harvested and made electrocompetent. A PCR fragment containing the kanamycin gene flanked by 60bp of viral sequences on both sides was used for recombination to construct
BAC36ΔK8.1, containing the kanamycin cassette within the targeted K8.1 genomic region. Briefly, 40µl of electrocompetent DH10B cells, harboring both BAC36 and pGETrec, were electroporated with 200ng of purified PCR product to delete the target gene (K8.1 glycoprotein) using standard electroporation parameters (1.8kV/cm, 200Ω and 25µF). Following electroporation, cells were grown in 1ml of LB for 60min and subsequently streaked onto LB agar plates containing both chloramphenicol (12.5 µg/ml) and kanamycin (50µg/ml). Episomal mutant BAC36 DNA containing the deletion in either the K8.1 gene via insertion of the kanamycin cassette was isolated from bacteria colonies and a second round of electroporation was performed to remove the plasmid pGETrec and grown on agar plates with chloramphenicol and kanamycin.

**Confirmation of the K8.1 Deletion in BAC36ΔK8.1 DNA**

KSHV BAC DNAs (BAC36 and BAC36ΔK8.1) were purified from 1 liter of BAC cultures using the Qiagen Large-Construct Kit (Qiagen; Valencia, CA). BAC DNA was digested with KpnI and run on 0.8% agarose gels, and the restricted DNA was transferred to charged nylon membranes (BioRAD; Richmond, CA). Southern-blot hybridization was performed using a biotin-labeled kanamycin resistance gene probe by labeling a 1.1kb kanamycin PCR fragment with biotin (New England Biolabs; Boston, MA). Chemiluminescence detection of the DNA was performed using the North2South Chemiluminescent Hybridization and Detection Kit as described by the manufacturer (Pierce Inc.; Rockford, IL).

**Transient Transfection of KSHV BAC DNAs**

Transient transfection of 293 cells with BAC DNAs was performed using Superfect (Qiagen; Valencia, CA). 293 cells were grown to 80% confluency in 6 well plates. Cells
were transfected with BAC DNA mixed with Superfect in DMEM media as recommended by the manufacturer (Qiagen). After 4 hours of incubation at 37°C, the media was removed from the transfected cells and washed with PBS and subsequent fresh DMEM media with 10% fetal calf serum was added.

**Immunohistochemical Staining**

293 cells in 6 well plates were transfected with BAC36 or BAC36ΔK8.1 DNA either alone or cotransfected with the Rta plasmid for induction of the lytic cycle. Forty-eight hours after transfection, cells were washed with PBS and fixed with electron microscopy-grade 2% paraformaldehyde (Electron Microscopy Sciences; Fort Washington, PA) for 10 min, and then washed twice with phosphate-buffered saline-50mM glycine. Blocking of cell monolayer for 1 hour was performed using 5% normal goat serum and 5% bovine serum albumin in PBS. Cells were then incubated for 1 hour with a primary antibody, washed three times with PBS, and then incubated for 1 hour with a secondary antibody conjugated with biotin. Cells were washed three times with PBS, and the cells were then reacted with a 1:3000 dilution of hrp-streptavidin in 10% goat PBS for 1 hour. Cells were washed five times and substrate from Vector VIP was added (Vector Laboratories Inc.; Burlingame, CA). Cells were examined using a light microscope (Nikon Inc; Garden City, NY). Primary antibodies used in these studies were: anti-K8.1 (19b4;(Wu et al., 2000)), anti-ORF59, and anti-LANA antibody (Advanced Biotechnologies, Inc, Columbia, MD).

**Production of Infectious KSHV Particles**

293 cells were transfected with either BAC36, BAC36ΔK8.1 or EGFP-C1 DNA each mixed with the ORF50 (RTA) and either pCDNA3.1 (negative control) or pCDNAK8.1
(complementing plasmid) in 6 well plates. Transfected cells were induced with a final concentration of 25ng/ml of tetradecanoyl phorbol acetate (TPA) (Sigma; St. Louis, MO) and 1,000 units/ml of Interferon-α (Sigma) for 5 days. Supernatant was collected from induced cell lines and centrifuged three times at 5,000g for 15min. Supernatants were freeze-thawed three times to eliminate any surviving cells. The viral supernatant was then used for infection or quantitative PCR analysis.

**Infection of Cells with Harvested Supernatants**

Supernatants from transiently transfected cells were used as viral inoculum to infect cells in a 96 well plate at 80% confluency. Infection was performed in triplicate; 50µl of the infectious viral inoculum with Polybrene added to a final concentration of 5 µg/ml (Sigma; St. Louis, MO) was placed on 293 cells for five hours and then 50µl of fresh media was added and the plate was incubated overnight at 37˚C. The next day, the viral inoculum was removed and fresh media was added. Infectivity was determined two days postinfection by counting the number of green fluorescent protein (GFP)-expressing cells via fluorescence microscopy as described previously (Gao, Deng, and Zhou, 2003).

**Sample Preparation for KSHV Quantitative TaqMan PCR**

293 cells were co-transfected with various combinations of KSHV BAC genomes and plasmids pRTA, pCDNA3.1 and pCDNAK8.1. All transfections contained the same amount of total DNA transfected. The amount of BAC36 or BACΔK8.1 remained constant, while varying amounts of pCDNA3.1 (control), the RTA expressing plasmid and the pCDNAK8.1 complementing plasmid were used. TPA (25ng/ml) and interferon-α (1000 units/ml) were added 24 hours post-transfection to enhance viral induction. The supernatants were centrifuged three times at 5,000g for 15 minutes to remove floating
cells. The supernatants collected after centrifugation were subsequently treated with TurboDNase (Ambion Inc; Austin, TX), for three hours at 37°C to ensure that unencapsidated genomes were not present during the real-time PCR assay. In these experiments, one unit of TurboDNase was used per 100 µl of sample. In control experiments, one unit of TurboDNase reduced BAC36 DNA by more than one thousand fold as determined by TaQman PCR. Viral DNA from supernatants was extracted in triplicate using a standard Proteinase K, phenol/chloroform protocol.

**KSHV TaqMan PCR Quantitation**

Reagents and enzymes used for TaqMan PCR were obtained from PE Applied Biosystems (Foster City, CA). The sequence for the TaqMan FAM-probe and primers to ORF37 used for the quantitative detection of KSHV molecules were published previously (Stamey et al., 2001) and listed in Table 1. Each 25 µl PCR contained a 1X TaqMan universal PCR master mix, 0.25 µl of 20 µM Primer stock for both forward and reverse primers. The 100 µM ORF37-FAM labeled probe was diluted 1:50 and 0.25 µl of probe was used per reaction. The reaction conditions were as followed: 2 minutes at 50°C, 10 min at 95°C with a subsequent 40 cycles two-step PCR (95°C for 15 sec, 60°C for 1 min). During amplification, an ABI-prism 7700 sequence detector monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. Samples were analyzed in triplicate on three independent runs. The C<sub>t</sub> value is defined as the cycle number in which the fluorescence detected exceeded an established threshold level that was kept constant in all experiments (Heid et al., 1996). The concentration of the BAC36 DNA after purification using the Large Construct Kit (Qiagen; Valencia, CA) was determined by optical density at 260nm and comparative gel electrophoresis to known
amounts of molecular markers. Serial ten-fold dilutions of BAC36 DNA were used as controls to construct a standard curve based on the \( C_t \) value and logarithmic amounts of diluted BAC36. The number of KSHV genomes in each specific supernatant sample was determined by comparison of the obtained \( C_t \) value to corresponding values of the standard curve reflecting a specific amount of KSHV viral DNA. The number of genomes within this specific amount of viral DNA was obtained using the following relationship: one KSHV genome was approximately equal to 0.197 femtograms.

RESULTS

Construction of the KSHV BAC36\( \Delta \)K8.1

The complete KSHV genome was recently cloned into a bacterial artificial chromosome (BAC36) enabling the genetic manipulation of the KSHV genome in \( E. \ coli \). BAC36 constitutively expresses the GFP gene allowing detection of eukaryotic cells containing KSHV genomes (Zhou et al., 2002). The BAC-based GET homologous recombination system was utilized to construct a large deletion within the K8.1 gene in \( E. \ coli \). Deletion of most of the K8.1 gene was accomplished by targeting the K8.1 ORF using specific primers as detailed in the Materials and Methods section. The genomic region encompassing the K8.1 gene codes for the K8.1A and K8.1B via spliced transcripts. The 5’ most primer (primer A; Table 2.1, Figure 2.1A) used for homologous recombination overlaps the K8.1 ATG and extends into the K8.1 ORF by 6 nucleotides. Insertion of the kanamycin cassette inserted multiple stop codons in different frames immediately down stream of the ATG codon preventing the expression of any aberrant proteins. The 3’ primer (primer B; Table 2.1, Figure 2.1A) is approximately 90 nucleotides upstream of the TAA stop codon of K8.1 (Figure 2.1B).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Purpose &amp; Prod. Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 5'K8.1/KanF</td>
<td>5'--CAATATTTAAGGGAGCCAAGGTTAATCCCTTAAT</td>
<td>GET-CTCTGGGATTAATAACCATGAGTTAGCCACGTG TGTCTCAAAATCTCTGATGTTA--3' (1.2kb)</td>
</tr>
<tr>
<td>B: 3'K8.1/KanR</td>
<td>5'--CTAGCACAGGTAAAGTATAAGGACAAGTCCACGC AATAAACCACAGCCATAGTGTACGGTTGATGA GAGCTTTGTGTAGTTGGAC--3'</td>
<td></td>
</tr>
<tr>
<td>C: 5'K8.1/up</td>
<td>5'--CATGCTGATGCGAATGTGCA--3'</td>
<td>Diagnostic PCR (wt) BAC36,</td>
</tr>
<tr>
<td>D: 3'K8.1/nostop</td>
<td>5'--CACTATGTAAGGTTTCTTACG--3'</td>
<td>BAC36ΔK8.1;(1.4kb)</td>
</tr>
<tr>
<td>E: 5'KanFwd</td>
<td>5'--ATGAGCCATATTCAACCG--3'</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>F: 3'KanRev</td>
<td>5'--CTCATCGAGCATCAAATG--3'</td>
<td></td>
</tr>
<tr>
<td>G: 5'ORF37-Fwd</td>
<td>5'--TCGGTGCGATGCTTTAGAC--3'</td>
<td>Taqman product</td>
</tr>
<tr>
<td>H: 3'ORF37-Rev</td>
<td>5'--TGAAGCAGACGATGCTTTGC--3'</td>
<td></td>
</tr>
<tr>
<td>I: RF37-Fam-probe</td>
<td>5'--TCGTAACCCCCTCCTACCTTTCCCCG--3'</td>
<td>Taqman FAM-</td>
</tr>
<tr>
<td>J: K8.1F</td>
<td>5'--TAACCATGAGTCCACACAGATTC--3'</td>
<td>K8.1A</td>
</tr>
<tr>
<td>K: K8.1R</td>
<td>5'--GGTTTTGTGTACACTATGTAGAG--3'</td>
<td></td>
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Figure 2.1: Schematic representation. (A) Schematic illustration of the GET homologous recombination BAC36ΔK8.1 DNA. A PCR fragment containing the kanamycin resistance gene flanked by 60bp of the K8.1 upstream and 3’end sequences was used to construct BAC36ΔK8.1 DNA recombinants containing the kanamycin resistance gene cassette within the targeted genomic site. The primer binding sites of the majority of the primers (Table 2.1) used to create the BAC36ΔK8.1 DNA are as shown. (B) Schematic representation of the ORF 50 gene locus and the relevant transcript coding for ORF 50 as presented in detail previously (West and Wood, 2003). Vertical dashed lines indicate the deleted genomic region encompassing most of the K8.1 ORF. The relative location of each gene is indicated as well as the location of splice donor (SD) and splice acceptor (SA) sites are indicated.
The BAC36ΔK8.1 construct was tested for the presence of the engineered insertion via diagnostic PCR and Southern-blotting (Figure 2.2). Primers C and D (Table 2.1, Fig. 2.1A) located immediately upstream of the K8.1 gene and within the 3’ undeleted portion of the K8.1 ORF, respectively, were used to amplify a diagnostic DNA fragment from KSHV DNA purified from BCBL-1 cells, BAC36 DNA and BAC36ΔK8.1 DNA (Figure 2.2A). DNA from BCBL-1 cells and BAC36 produced a predicted DNA fragment of 964 bp. In contrast, amplification of the targeted region of the BAC36ΔK8.1 DNA produced a DNA fragment of approximately 1.4 kbp as predicted after insertion of the kanamycin gene cassette. Further confirmation of the genetic content of the BAC36ΔK8.1 was obtained via restriction endonuclease fragment analysis and Southern-blotting (Figure 2.2B). Restriction enzyme analysis of BAC36 and BAC36ΔK8.1 DNA with KpnI revealed a similar DNA fragmentation pattern with the exception that a DNA fragment of approximately 4.6 kbp was absent in the BAC36ΔK8.1 profile in comparison to the BAC36 restriction pattern. In addition, the BAC36ΔK8.1 pattern included a new DNA fragment migrating with an apparent molecular mass of approximately 5 kbp in agreement with the theoretical size of a new DNA fragment produced after insertion of the kanamycin gene cassette within the K8.1 gene (Figure 2.2B: panel 1). Southern-blotting using a biotinylated kanamycin gene probe of KpnI digested BAC36 and BAC36ΔK8.1 DNA revealed the presence of a unique kanamycin insertion within a DNA KpnI fragment of approximately 5 kbp, while there was no reaction of the probe with the BAC36 DNA. A pre-biotinylated molecular weight ladder was used for DNA fragment size determination (Figure 2.2B: panel 2). These results are consistent with the insertion
Figure 2.2: Genomic analysis of BAC36ΔK8.1 DNA. (A) PCR assay to confirm the deletion of the K8.1A gene in BAC36. Amplification of the K8.1 region produced a 964bp band from both BCBL-1 and BAC36 DNAs; the mutant BAC36ΔK8.1 DNA showed a band of 1405 bp due to the size difference in the deletion/insertion mutagenesis. Lane M, molecular size markers. (B: Panel 1) Restriction fragment analysis of BAC36ΔK8.1 DNA and wild-type BAC36 DNA. The KpnI restriction pattern of BAC36ΔK8.1 DNA was compared to that of wild-type BAC36 DNA. As predicted from the KpnI restriction pattern, the introduction of the kanamycin resistance gene into BAC36 led to an increase in size from an estimated 4.5kb fragment to a 4.9kb fragment (arrow). (B: Panel 2). Southern-blot analysis of BAC36ΔK8.1 DNA. The KpnI restriction fragment profile from panel 1 was hybridized with a probe derived from a kanamycin PCR product that was labeled with biotin. A biotinylated kanamycin probe hybridized to an estimated 4.9kb KpnI DNA fragment in BAC36ΔK8.1 KpnI restricted DNA, which is absent in the wild-type BAC36 DNA (arrow).
of the kanamycin gene cassette within the K8.1 gene and deletion of most of the K8.1 ORFs.

**Transient Expression of K8.1A**

The K8.1 gene was cloned into the transient expression plasmid pCDNA3.1 and expressed in 293 cells by transfection. Detection of the transiently expressed K8.1A glycoprotein was achieved via indirect immunofluorescence utilizing anti-K8.1 mAb 19B4 (Figure 2.3A: panel A) (Wu et al., 2000). Approximately, 50% of the transfected cells reacted strongly with the anti-K8.1 19B4 mAb, while mock-transfected cells failed to react in immunofluorescence assays (Figure 2.3A: panels B, D). Similar results were produced via immunohistochemical staining of 293 cells transfected with plasmid pCDNA3.1-K8.1A (Figure 2.3A: panel C). To further characterize expression of K8.1A under transient expression conditions, lysates of 293 transfected cells were electrophoretically separated in SDS-PAGE and tested via immunoblotting using mAb 19B4 and compared to K8.1 expressed in BCBL-1 cells after TPA induction (Figure 2.3B). Transiently expressed K8.1A migrated with an apparent molecular mass of 37 kDa, while BCBL-1 cells expressed K8.1 migrating with molecular masses ranging from 34-37 kDa (Figure 2.3B: lanes 2, 3). The 37 kDa glycoprotein species has been shown to represent fully glycosylated K8.1 glycoprotein (Raab et al., 1998). The 19B4 mAb reacted with proteins of higher apparent molecular masses presumably corresponding to higher order multimers of K8.1 (Wu et al., 2000).

**Characterization of Latent and Lytic Gene Expression**

Log-phase 293 cells were transfected with BAC36 or BAC36ΔK8.1 DNA and expression profiles of the latent gene LANA and the lytic genes ORF59 and K8.1 were
Figure 2.3: Detection of K8.1A gene expression in transiently transfected cells. (A) Immunofluorescence (panel A) and immunohistochemical staining (panel C) of K8.1A transiently transfected 293 cells. Immunofluorescence (panel B) and immunohistochemical reactivities (panel D) of mock-transfected 293 cells. (B) Immunoblot of cell lysates from mock-transfected cells (lane 1), cells transiently transfected with K8.1A (lane 2), and induced BCBL-1 cells (lane 3).
assessed via immunohistochemical staining using mAbs specific for each protein (Figure 2.4). Typically, the BAC36ΔK8.1 DNA transfected more cells (20-30%) versus the BAC36 DNA (10-15%). In the absence of TPA induction, the anti-LANA antibody reacted with cells transfected with either BAC36 or BAC36ΔK8.1 DNA (Figure 2.4, panels F, L). Similar results were obtained after the transfected 293 cells were induced with TPA (Figure 2.4, panels I, O). Immunostaining of 293 transfected cells with the anti-ORF59 antibody detected expression of ORF59 (lytic gene) in either the absence (Figure 2.4: panels E, K) or presence (Figure 2.4: panels H, N) of TPA induction, although after TPA induction, approximately 2-fold more cells stained positive for ORF59. Similar results were obtained with the anti-K8.1 mAb immunostaining with the exception that K8.1A was not expressed in 293 cells transfected with the BAC36ΔK8.1 DNA (Figure 2.4: panels J, M). No reactivity was observed by the anti-K8.1, anti-ORF59 or the anti-LANA antibodies against the 293 mock-transfected negative control cells (Figure 2.4: panels A, B, C).

**Production of Infectious KSHV by BAC36 and BAC36ΔK8.1 Transfected 293 Cells**

To assess the role of K8.1 in viral infectivity, purified BAC36 and BAC36ΔK8.1 DNAs were co-transfected with an RTA plasmid transiently expressing the RTA (ORF50) gene and either pCDNA3.1 (control) or pCDNAK8.1 (complementing plasmid). The number of transfected cells expressing GFP remained constant in the presence or absence of the RTA expressing plasmid (not shown). RTA is the major KSHV transactivator protein capable of inducing lytic replication of KSHV (Sun et al., 1998). KSHV lytic replication was further enhanced through the addition of TPA and interferon-α for five days, at which point supernatants of transfected cells were clarified of cellular debris and tested
Figure 2.4: Latent and lytic gene expression profiles of uninduced and induced 293 cells transfected with either BAC36 DNA or BAC36ΔK8.1 DNA. Transfections with BAC36ΔK8.1 DNA did not result any expression of a K8.1A gene product after RTA and TPA induction (panels J and M). As a positive control, transiently transfected 293 cells with BAC36 DNA reacted with the K8.1A antibody (panels D and G). The anti-ORF59 mAb reacted with uninduced or induced (TPA plus RTA) 293 cells transfected with either BAC36ΔK8.1 (panels K and N) or BAC36 DNA (panels E and H). Similarly, the anti-LANA mAb reacted with both uninduced and induced BAC36 DNA (panels F and I, respectively) or BAC36ΔK8.1 DNA (panels L and O, respectively) transfected 293 cells. Mock-transfected 293 cells failed to show any reactivity with either anti-K8.1, anti-ORF59 and anti-LANA mAbs (panels, A, B and C, respectively). Arrows indicate K8.1, ORF59, or LANA expression in 293 cells.
for the presence of infectious KSHV. The relative infectivity of supernatant viruses was determined by monitoring the number of GFP-expressing cells (see Materials and Methods) after infection of 293 cells (Gao, Deng, and Zhou, 2003) (Figure 2.5: panels A, B, C). Control experiments were also performed in parallel to determine whether cellular debris from transfected cells carried over the GFP during the infection process. 293 cells were transfected with plasmids pRTA and pCDNA3.1 and the pEGFP-C1 constitutively expressing GFP, and extracellular fluids were treated similarly to those containing infectious virus and tested for their ability to produce GFP-expressing cells under similar infection conditions. These control experiments revealed no GFP carry over from ruptured cells (Figure 2.5A: panel D). Transfection experiments performed in triplicate revealed that BAC36 and BAC36ΔK8.1 DNAs consistently produced on the average 260 and 460 GFP-expressing cells/ml, respectively. Co-transfection of BAC36ΔK8.1 DNA with the complementing pCDNA-K8.1A plasmid seemed to enhance infectious virus production by approximately two-fold (Figure 2.5B). Co-transfection of BAC36 DNA with pCDNA-K8.1 or other control plasmids did not have an effect on infectious virus production. Furthermore, different isolates of BAC36 or BAC36ΔK8.1 exhibited similar infectivities (data not shown).

Quantitation of KSHV via TaqMan PCR

To better quantify the amount of virus produced by transfection of 293 cells, a real-time PCR assay targeting the ORF37 gene was employed essentially as described previously (Stamey et al., 2001). The relative amounts of BAC36 DNA and BAC36ΔK8.1 DNA found in supernatants of 293 transfected cells were obtained from a standard curve based on known amounts of BAC36 DNA (see Materials and Methods).
Figure 2.5: Relative infectivity of KSHV mutants. 293 cells were infected with virus obtained from supernatants of 293 cells transfected with BAC36 and BAC36ΔK8.1 DNA. (A) Approximately $10^4$ 293 cells per well in a 96 well plate were incubated with 50µl of supernatant containing virus obtained from transfections of 293 cells with BAC36, pRTA and pCDNA3.1 (panel A2), BAC36ΔK8.1, pRTA and pCDNA3.1 (panel B2), BAC36ΔK8.1, pRTA and pCDNAK8.1 plasmid (panel C2), or pEGFP-C1, pRTA and pCDNA3.1 (panel D2). Matching phase contrast images are shown (panels: A1, B1, C1, D1). (B) Virus infectivity was determined by the number of GFP-expressing cells. Error bars indicate standard deviations. All experiments were performed in triplicates. Infectious virions were obtained from 293 cells transfected with DNA mixtures as shown.
All BAC-transfected 293 cells produced relatively high number of genome equivalents in supernatants (Figure 2.6). The number of BAC36ΔK8.1 genomes found in supernatants of transfected cells was consistently higher than that of BAC36, in part due to the higher transfection efficiency of the BAC36ΔK8.1 DNA over the BAC36 DNA (data not shown). Cotransfection of the pCDNA-K8.1A plasmid with BAC36ΔK8.1 produced approximately two-to-three fold more viral genomes in 293 cellular supernatants than did transfection BAC36ΔK8.1 alone (Figure 2.6). This effect was pCDNAK8.1-specific, since co-transfection of BACΔK8.1 DNA with vector plasmid pCDNA3.1 did not increase the number of viral genomes found in supernatants (Figure 2.5B).

Viral genomes in supernatants were protected from TurboDNase treatment indicating that most of the viral genomes in 293 supernatants were encapsidated (Figure 2.6). These results were most likely not due to differences in transfection efficiencies, since the number of transfected cells expressing the viral GFP gene remained approximately constant in the presence or absence of the complementing pCDNA-K8.1A plasmid (data not shown). Therefore, the two-to-three fold increase of encapsidated KSHV(ΔK8.1) genomes in the supernatants from cells transfected with pCDNA-K8.1A, when compared to transfections with BAC36ΔK8.1 alone, is probably due to the production of a greater number of encapsidated KSHV virions (Figure 2.6).

**DISCUSSION**

Viral glycoproteins encoded by herpesviruses are important determinants of viral infectivity and spread. Although there are significant differences among herpesviruses belonging to the three herpesvirus subfamilies: alpha, beta and gamma, there are important similarities among all herpesviruses with regard to virus entry and egress out of
Figure 2.6: Quantitative, real-time PCR assay determination of KSHV genomes in supernatants of transfected 293 cells. Supernates were either treated (gray, striped bars) or not treated (black, solid bars) with DNase I. Viral DNA extraction and real-time PCR were performed in triplicate from supernatants obtained from cells transfected with either BAC36, BAC36ΔK8.1, or BAC36ΔK8.1 DNAs and various plasmid mixtures. Supernatants from 293 cells transfected with pEGFP-C1, pCDNA3.1, and pRTA plasmids were included as a negative control. Error bars indicate standard deviations.
infected cells. Generally, all herpesviruses require initial attachment onto cell surfaces, often mediated by binding to ubiquitous glycosaminoglycans such as heparan sulfate. Virus penetration is achieved via either direct fusion of viral envelopes with cellular membranes or after virion endocytosis and subsequent fusion of viral envelopes with endosomal membranes releasing capsids into the cytoplasm of infected cells. For KSHV, initial attachment onto cell surfaces is mediated by binding of glycoproteins gB and K8.1 onto heparan sulfate moieties (Akula et al., 2001a; Akula et al., 2001b; Birkmann et al., 2001; Wang et al., 2001). In addition, gB binds to the α3β1 integrin receptor, which is thought to facilitate virus penetration into certain cells via endocytosis (Akula et al., 2002). The role of K8.1A in virus infectivity is not immediately apparent, since an initial report indicated that soluble K8.1A substantially inhibited virion attachment onto cells (Wang et al., 2001), while a later report suggested that a similar soluble form of K8.1A did not inhibit virus infectivity (Birkmann et al., 2001). In this report, we directly addressed the role of K8.1 in virus infectivity by generating a KSHV recombinant virus with a deletion of both spliced variants of the K8.1 gene. Characterization of this mutant virus in 293 cell culture assays revealed that the K8.1 gene were not essential for virus entry.

The BAC36ΔK8.1 virus was made using the GET homologous recombination system to insert a gene cassette coding for kanamycin resistance within the BAC36 K8.1 genomic region. The K8.1 gene deletion spanned the K8.1 ORF immediately after the initiation codon (ATG) and extended to approximately 90 bases upstream of the K8.1 termination codon. KSHV ORF50 specifying the major lytic transactivator RTA as well as the K8 ORF specifying the K-bZIP gene, which is suggested to act either as a
repressor of lytic replication (Izumiya et al., 2003; Liao et al., 2003) or as a replication activator protein (rap) (Wu et al., 2001), lie immediately 5’ to the K8.1 gene. All three genes code for mRNAs that utilize the same polyadenylation signal immediately downstream of K8.1. The engineered deletion of K8.1 removes the last splice acceptor site and consequently, splicing of the 3’ most exon. It is possible that the resultant 3’ modification of the RTA and K8 mRNAs adversely affected expression of these proteins. To ensure that the transfected BAC36ΔK8.1 DNA could enter into lytic replication, a plasmid capable of transient expression of RTA was co-transfected with the BAC36ΔK8.1 DNA. Consistently, the BAC36ΔK8.1 DNA produced 3-5-fold more infectious virus than BAC36 DNA. A possible explanation for this result may be that deletion of the terminal exon of K-bZIP (K8) reduced its expression allowing for improved trans-activation by the exogenously provided RTA.

KSHV genomes remain latent in BCBL-1 cells and could be activated to lytic replication as evidenced by the expression of lytic proteins such as ORF59 and K8.1 after induction with TPA and/or exogenously provided RTA. Transfection experiments in 293 cells revealed that both BAC36 and BAC36ΔK8.1 genomes expressed a low level of ORF59 indicating that under transient transfection conditions some of the viral genomes may be activated for lytic replication in the absence of exogenously provided TPA or RTA, which is consistent with previous observation (Zhou et al., 2002). These results are also in agreement with recent observations that infection of primary human umbilical vein endothelial cell (HUVEC) cultures occurred in two phases: 1) a permissive phase, in which the cultures undergo active viral lytic replication and infectious virus production; 2) a latent phase, in which the surviving cells from the lytic phase switch into latent
infection, with a small number of cells undergoing spontaneous viral lytic replication (Gao, Deng, and Zhou, 2003). However, in most experiments, trans-activation by RTA produced at least 2-3 fold more cells in which KSHV replicated in a lytic phase as evidenced by the expression of the ORF59 and K8.1 genes. As expected, the BAC36ΔK8.1 failed to express any K8.1 under either induced or uninduced conditions.

Transfection of either BAC36 or BAC36ΔK8.1 DNA into 293 cells produced infectious virions in the supernatants of the transfected cells. The number of infectious virions produced by the BAC36ΔK8.1 DNA transfection in triplicate shown in Figure 5B was higher than that of BAC36 DNA. This difference was largely due to lower transfection efficiencies obtained with BAC36 DNA (approximately 15% of 293 cells expressed GFP) in comparison to BAC36ΔK8.1 DNA (approximately 30% of 293 cells expressed GFP) as determined by the number of GFP-expressing cells (not shown).

Cotransfection of the BAC36ΔK8.1 DNA with plasmid pCDNA-K8.1A, transiently expressing the K8.1 glycoprotein, consistently produced approximately two-fold higher numbers of infectious virions in extracellular fluids in comparison to transfections with BAC36ΔK8.1 DNA with pCDNA3.1 (control). This result was not due to differences in transfection efficiencies between BAC36ΔK8.1 with pCDNA3.1 DNAs and BAC36ΔK8.1 with pCDNAK8.1 DNAs because both transfections produced equivalent numbers of GFP expressing 293 cells. In addition, supernatants from 293 cells transfected with a plasmid expressing GFP (pEGFP-C1) with pCDNA3.1 did not produce any GFP positive cells indicating that there was no GFP carry over by potentially ruptured cells. These results indicate that K8.1 is not needed for virus entry, since BAC36ΔK8.1 virions remain infectious in the absence of K8.1.
To differentiate the complementation effect of transiently expressed K8.1 on BAC36ΔK8.1 infectious virus production, the number of KSHV genomes contained in extracellular fluids of transfected 293 cells was determined via real-time PCR. Co-transfection of BAC36ΔK8.1 with the complementing plasmid pCDNA-K8.1 consistently produced higher number of viral genome equivalents in supernatants in comparison to BAC36ΔK8.1 with pCDNA3.1. These genomes represented encapsidated DNA since treatment with DNase I did not appreciably reduce the number of KSHV genomes suggesting that K8.1 enhanced infectious virus production.

Previous studies addressing the role of K8.1A in virus attachment showed that a soluble version of K8.1A reduced KSHV attachment by as much as 70% (Wang et al., 2001). In a latter study, a similar soluble form of K8.1A did not affect virus infectivity (Birkmann et al., 2001). In this report, we conclusively show that K8.1 is not necessary for virus infectivity, in agreement with the latter study mentioned above. The relative increase of encapsidated KSHV virions after transient expression of K8.1 suggest that K8.1 may facilitate virion morphogenesis. A similar scenario may be occurring for the EBV gp350/220, since deletion of this gene caused a decrease in infectivity, which was complemented in-trans by exogenously supplied gp350/220 (Janz et al., 2000).

Characteristically, KSHV produces a high number of non-infectious virions in supernatants of BCBL-1 cells after induction to lytic replication. In one study, it was calculated that approximately $1 \times 10^7$ genome equivalents were produced per 1 ml of BCBL-1 suspension culture at 48 hours post TPA induction (Renne et al., 1996). However, typically, a very small portion of these viruses enters into susceptible cells and initiate a viral infection as evidence by the expression of either latent or lytic genes
transfections produced a relatively high number of encapsidated genomes in the supernatants of transfected cells; however, a very small percentage of these virions were infectious as evidenced by the expression of the virally encoded GFP gene after infection of 293 cells. It is unclear at this time why KSHV produces such a high number of noninfectious viruses, which are presumably defective in one or more functions. One possibility is that 293 cells do not support efficient cytoplasmic morphogenesis and egress resulting in a high percentage of partially defective virions in supernatant fluids. Production of high titer infectious KSHV should enable the ultrastructural visualization of egressing KSHV and enable visualization of the intracellular sites utilized by the virus as well as dissection of the role of individual glycoproteins in efficient morphogenesis and egress.

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CHAPTER III

CONSTRUCTION AND CHARACTERIZATION OF RECOMBINANT STRAINS USING THE BAC36 VIRAL GENOME SUGGEST THAT THE GLYCOPROTEIN B (gB) CARBOXYL-TERMINUS IS INVOLVED IN VIRUS-INDUCED CELL-TO-CELL FUSION

INTRODUCTION

The Human Herpesvirus 8 (HHV-8) or Kaposi’s Sarcoma-Associated Herpesvirus (KSHV) was first identified in 1994 by Patrick Moore and Yuan Chang (Chang et al., 1994). KSHV is considered to be the etiologic agent of Kaposi’s Sarcoma, an angiogenic vascular cancer of endothelial cells, which is more prevalent in HIV patients (Ablashi et al., 2002; Dourmishev et al., 2003; Martin and Osmond, 1999). The KSHV genome derived from the BC-1 cell line was fully sequenced in 1996 by the Moore/Chang Laboratory (Moore et al., 1996). KSHV is a member of the herpesviridae family, which include herpes simplex 1 & 2 (HSV-1 & 2), VZV (HHV-3), human cytomegalovirus (HHV-5), and the Epstein-Barr virus (HHV-4) (Knipe et al., 2001).

KSHV expresses numerous glycoproteins during its virus lifecycle. Many of these KSHV glycoproteins play important roles in virus entry and cell-to-cell spread (Knipe et al., 2001). KSHV glycoprotein B (gB) is conserved among all herpesviruses and is thought to be directly involved in fusion of the viral envelope with cellular membranes during virus entry, as well as in virus-induced cell fusion mediated by expression of gB and other viral glycoproteins on infected cell membranes. Virus-induced cell fusion allows herpesviruses to spread to adjacent cells avoiding the immune system (Baghian et al., 1993; Foster, Melancon, and Kousoulas, 2001). gB is a structural component of the KSHV virion particle (Akula et al., 2001; Baghian et al., 2000), and it is thought to
mediate similar membrane fusion functions to the other herpesviral gB homologues. One
unusual property of both herpes simplex virus type 1 (HSV-1) and KSHV gB is that
carboxyl terminal mutations cause enhanced virus-induced cell fusion (Baghian et al.,
1993; Foster, Melancon, and Kousoulas, 2001; Pertel, 2002). Specifically, a carboxyl
terminal deletion of KSHV gB was reported to cause increased amount of cell fusion in a
transient expression system where KSHV gB was co-expressed with gH and gL (Pertel,
2002). This gB truncation was not congruent to other HSV-1 gB truncations that were
previously shown to produce extensive virus-induced cell fusion, as well as cell-to-cell
fusion after transient co-expression of HSV-1 gB, gD, gH and gL. Based on the
conserved homology of the gB tail throughout the entire herpesvirus family, we
hypothesize that the carboxy-tail of KSHV gB plays a crucial role in gB’s ability to cause
membrane fusion.

Previously, classical methods for producing KSHV mutant viruses prevented the
rapid isolation of recombinant viruses containing specific mutations. In an effort to
delineate and map the functional domains of the gB carboxyl terminus, which are
involved in KSHV-induced cell fusion, a two-step BAC SacB mutagenesis method was
employed using the KSHV genome cloned into BAC36 (Gong et al., 2002; O'Connor,
Peifer, and Bender, 1989; Posfai et al., 1997; Smith and Enquist, 1999). In addition, a
SacB negative-selection process was applied to facilitate the rapid isolation of KSHV
mutant genomes. The two-step SacB mutagenesis system was used to insert two different
opal stop codons in the carboxy-tail of KSHV-gB resulting in carboxyl terminal
truncations: 1) gB-Pert; Ala 730 switched to opal stop codon 730, thus truncating the gB
carboxyl tail by 58 amino acids. 2) gB-Gus; Gly 749 switched to opal stop codon 749;
thus truncating the gB carboxyl tail by 25 amino acids. The ability of these recombinant
viruses to induce different amounts of virus-induced cell fusion is currently under
investigation.

**MATERIAL AND METHODS**

**Construction of Recombinant Plasmids Encoding Mutant gBs**

Mutagenesis of BAC36 DNA was accomplished in *E. coli* via the SacB/RecA
two-step mutagenesis system. PCR using primers IX and X amplified the KSHV-gB
region (4.6kb), which included the entire gB-gene, 1 kb upstream viral sequence (arm A)
tagged with SacII and 1 kb downstream viral sequence (arm B) tagged with NotI. The
PCR product was digested with SacII and NotI and cloned into the similarly restricted
plasmid, pSacB/RecA. The newly constructed plasmid, named pSacB/RecA-gB, served
as the gB homologous recombination vector and was used as the template to insert the
gB-carboxyl tail mutations: gB-Pert and gB-Gus. The pSacB/RecA-gB vector, in addition
to containing the 4.6kb gB-region, contained the R6Kγ origin, the E. coli RecA gene, a
SacB gene, and an ampicillin-resistance gene.

The insertion of the gB-Pert mutation within the pSacB/RecA-gB plasmid was
performed by PCR overlap extensions of two individual PCR products which inserted a
stop codon and a *Bam*HI site within the gB-carboxyl-tail. Two PCR products which
constituted a 5’-end product (Primers IX & IV; Table 3.1) and a 3’-End product (Primers
III & X; Table 3.1) were cut with *Bam*HI and subsequently ligated to each other. A final
round of PCR (Primers IX and X; Table 3.1) was performed to amplify the mutated gB-
Pert region including the entire gB-gene, 1 kb upstream arm A and 1 kb downstream arm
B sequences; the final PCR product was subsequently cloned into pSacB/RecA via the SacII and NotI restriction sites, thus producing the pSacB/RecA-gB-Pert vector.

A similar strategy was employed for the insertion of the gB-Gus carboxyl tail mutation within the pSacB/RecA-gB plasmid. The insertion of the gB-Gus mutation was performed by PCR overlap extensions of a 5’-End-product (Primers IX & II; Table 3.1) and a 3’-End-product (Primers I & X; Table 3.1). The two PCR products which constituted a 5’-End product (Primers IX & II; Table 3.1) and a 3’-End product (Primers I & X; Table 3.1) were cut with BamHI and subsequently ligated to each other. A final round of PCR (Primers IX and X; Table 3.1) was performed which produced a PCR product which possessed an inserted stop codon and a BamHI site within the gB-carboxyl-tail. The final PCR product was subsequently cloned into pSacB/RecA via the SacII and NotI restriction sites, thus producing the pSacB/RecA-gB-Gus vector.

**Preparation of KSHV-BAC36 Competent Cells**

Overnight culture of E.coli DH10B cells harboring the KSHV-BAC36 genome was diluted 1000-fold in 100 mL of LB medium containing the antibiotic chloramphenicol (Chlor) (12.5µg/mL). The optical density was monitored at 600nm (OD600) to stop the growth of the bacteria at 0.7-0.8. The bacteria was chilled afterwards on ice for 15 min. The cells were then harvested and made electrocompetent by centrifuging and repeated resuspensions in 10% glycerol; the competent cells were aliquoted and stored at -80°C. The pSacB/RecA-gB-Pert (100ng) or pSacB/RecA-gB-Gus (100ng) was added to 40µl of chilled KSHV-BAC36 competent cells. Electroporation was performed with the BioRad Gene Pulser II with the following conditions: 1.8kV/cm, 200Ω and 25µF.
Identification of KSHV-BAC36-SacB/RecA-gB-Mutant Co-Integrates (Recombination Step 1)

Following electroporation, cells were grown in 1ml of SOC for 60 min at 37°C and subsequently diluted and grown in LB plates containing chloramphenicol (12.5 µg/ml) as well as ampicillin (100µg/ml) overnight at 37°C. The next day, colonies were picked and grown overnight in LB broth (chlor and amp) and the culture was further diluted 1:5,000 in LB broth (chlor and amp) and incubated for 10 hours at 37°C and the culture was diluted 1:5,000 once again for 8 hours at 37°C. Subsequently the selected bacteria were struck onto LB plates containing chloramphenicol (12.5 µg/ml) and ampicillin (100µg/ml) overnight at 37°C. Colonies were picked and grown overnight in LB broth (chlor and amp); the colonies were assayed by PCR for the integration of the gB-mutation recombination shuttle vector (pSacB/RecA-gB-Pert or pSacB/RecA-gB-Gus) into the KSHV-BAC36, thus forming a co-integrate (KSHV-BAC36-SacB/RecA-gB-Pert or KSHV-BAC36-SacB/RecA-gB-Gus).

Resolution of KSHV-BAC36-SacB/RecA-gB-Mutant Co-Integrates (Recombination Step 2)

The colonies, which were appropriately identified as a KSHV-BAC36-SacB/RecA-gB-Mutant co-integrates (KSHV-BAC36-SacB/RecA-gB-Pert or KSHV-BAC36-SacB/RecA-gB-Gus), were picked from LB-plates, containing chloramphenicol (12.5 µg/ml) and ampicillin (100µg/ml), and subsequently grown at 37°C for 4 hours in LB broth containing chloramphenicol (12.5 µg/ml) and 5% sucrose. The bacteria was diluted and grown overnight at 37°C onto LB plates containing 10% sucrose and chloramphenicol (12.5 µg/ml). Replica plating was employed for further screening; colonies were picked and plated onto two agar plates: 1.) LB-plates with chloramphenicol
(12.5 µg/ml) and ampicillin (100µg/ml) and 2.) LB-plates with only chloramphenicol (12.5 µg/ml). The colonies that grew only on plates with chloramphenicol (not the plates with double selection) were mini-prepped and screened by both restriction digest and PCR analysis. This resolution procedure produced both wild-type and mutant KSHV-BAC clones.

**Transient Transfection of the KSHV BAC DNAs**

Transient transfection of 293 cells with KSHV-BAC36 DNAs was performed by using Superfect (Qiagen). 293 cells were grown to 80% confluence in six-well plates. Cells were transfected with BAC DNA mixed with Superfect in DMEM as recommended by the manufacturer. After 4 hours of incubation at 37°C, the medium was removed from the transfected cells, and the cells were washed with PBS. Next, fresh DMEM with 10% fetal calf serum was added. In order to induce KSHV late gene expression within the KSHV-BAC transfected cells, TPA [12-O-Tetradecanoylphorbol-13-acetate] at (25 ng/ml) was added 24 h post-transfection.

**RESULTS**

**Construction of the pSacB/RecA-gB Homologous Recombination Plasmid**

More than a decade ago, our laboratory investigated the effect of several HSV-1 gB truncations on virus-induced cell fusion; the findings were clear, an HSV-1 recombinant virus expressing a 28 amino acid truncated gB resulted in extensive virus-induced cell fusion (Baghian et al 1993). In this study, we investigated KSHV virus-induced cell fusion by engineering an analogous truncation in the carboxyl tail of KSHV-gB, wherein we truncated the KSHV-gB molecule by 25 amino acids (gB-Gus) (Figure 123
TABLE 3.1. Synthetic Oligonucleotide Primers. The opal termination codon ‘TGA’ and its complementary sequence ‘TCA’ are in bold. Restriction sites are underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: 5'-Bam-Gus</td>
<td>5’—AGTATA<strong>TGA</strong>GATCCAAAAACGCTTCATCGCTCGTCTG—3’</td>
</tr>
<tr>
<td>II: 3'-Bam-Gus</td>
<td>5’—TTGTAT<strong>GATTT</strong>CATGCGGTACCTGAAACACCGA—3’</td>
</tr>
<tr>
<td>III: 5'-Bam-Pert</td>
<td>5’—CGTCTC<strong>TGA</strong>GATCCAAAAACATCGTGGGAATG—3’</td>
</tr>
<tr>
<td>IV: 3'-Bam-Pert</td>
<td>5’—ATAGAAG<strong>GATTT</strong>CATCCCGTGTTGGGGA—3’</td>
</tr>
<tr>
<td>V: 5'-F-Gus-Test</td>
<td>5’—ACGCGTACCGC<strong>TGA</strong>GATTC—3’</td>
</tr>
<tr>
<td>VI: 3'-R-Gus-Test</td>
<td>5’—ACGAAGGCGTGG<strong>GATTT</strong>C—3’</td>
</tr>
<tr>
<td>VII: 5'-F-Pert-Test</td>
<td>5’—CGGGAGGAAT<strong>TGA</strong>GATCC—3’</td>
</tr>
<tr>
<td>VIII: 3'-R-Pert-Test</td>
<td>5’—CAGGATTT<strong>TGG</strong>TG<strong>TCA</strong>—3’</td>
</tr>
<tr>
<td>IX: F-SacII-gBup</td>
<td>5’—ATATGACCGC<strong>AAT</strong>ACTACTGCACTGC—3’</td>
</tr>
<tr>
<td>X: R-NotI-gBdown</td>
<td>5’—AGTATA<strong>CGGGCGG</strong>GAGCCG—3’</td>
</tr>
</tbody>
</table>
3.1). A virus-free cell fusion assay has been developed for herpesviruses (Foster, Melancon, and Kousoulas, 2001; Muggeridge, 2000; Pertel, 2002; Pertel et al., 2001; Turner et al., 1998), particularly a KSHV virus-free cell fusion assay has been developed, in which transient transfection of KSHV-gB, gH and gL with gB having a 59 amino acid carboxyl terminal gene truncation induced cell-to-cell fusion (Pertel, 2002). In this study the ability of a KSHV-gB carboxyl tail truncation by 59 amino acids (gB-Pert), to induce cell-to-cell fusion was compared to other gB truncations (Figure 3.1).

To facilitate the insertion of the gB-carboxyl tail mutations (gB-Pert and gB-Gus) within the gB gene of the KSHV-BAC36 genome by homologous recombination in bacteria, a recombination vector (pSacB/RecA-gB) was engineered, in which the gB gene along with a 1 kb upstream homology arm ‘A’ and 1 kb downstream homology arm ‘B’, amplified using primers IX & X (Table 3.1), was cloned into the pSacB/RecA shuttle vector (Figure 3.2). The pSacB/RecA vector contains the versatile genetic elements which can complement the deficiency of DH10B E.coli for homologous recombination (see Materials and Methods) [1.) RecA gene, which mediates homologous recombination; 2.) SacB gene; a negative selection marker which allows for removal of any sequence remnants left from the homologous recombination system; 3.) R6kγ replication origin; can not replicate in DH10B E. coli (can only replicate in the presence of the π protein encoded by the pir gene for replication; 4.) Ampicillin resistance cassette; allows for positive selection of co-integrates, indicating the insertion of the shuttle vector into the KSHV-BAC36 genome].
Figure 3.1. A schematic representation of the predicted secondary structure of the cytoplasmic portion of KSHV glycoprotein B. This model depicts the gB carboxyl tail mutations named gB-Pert and gB-Gus. In this study, these gB-tail mutations were inserted within the wild-type KSHV-BAC36 producing the following mutant viruses: KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus. The relevant predicted α-helices are denoted as Tail Helix 1 and Tail Helix 2, which were marked by dashed lines. The schematic was based on the secondary structure prediction produced by using the PSIPRED Protein Structure Prediction Server. 
http://bioinf.cs.ucl.ac.uk/psipred/
Figure 3.2 Construction of pSacB/RecA-gB, a homologous recombination vector encoding KSHV-gB along with 1kb upstream (arm A) and downstream (arm B) overhang sequence. The overall strategy leading to the construction of the recombination plasmid, pSac-RecA-gB KSHV-BAC36-gB. (1), PCR amplification of the KSHV-gB region which includes 1 kb upstream viral sequence (arm A) tagged with SacII and 1 kb downstream viral sequence (arm B) tagged with NotI. (2) Restriction digestion of the PCR product and pSacB/RecA with SacII and NotI enzymes, which were subsequently ligated. (3) The gB homologous recombination cassette within the pSacB/RecA-gB was used as the basis for the subsequent insertion of gB-carboxyl tail mutations.
1.) PCR of gB w/ upstream arm A and downstream arm B.

2.) Restriction cut of PCR product and vector w/ subsequent ligation.

3.) gB recombination vector w/ upstream arm A and downstream arm B.
The pSacB/RecA-gB vector, which contained the genomic region encompassing the gB gene along with the 1kb upstream (arm A) and downstream sequences (arm B), was used to insert the gB-Pert and gB-Gus mutations within the gB gene of the KSHV-BAC36 genome. The overall strategy leading to the construction of the gB-mutated recombination plasmids (pSacB/RecA-gB-Pert and pSacB/RecA-gB-Gus) was performed by inserting within the gB carboxyl tail an Opal stop codon with a subsequent BamHI site: 1) gB-Pert; Ala 730 switched to opal stop codon 730, thus truncating the gB carboxyl tail by 58 amino acids. 2) gB-Gus; Gly 749 switched to opal stop codon 749; thus truncating the gB carboxyl tail by 25 amino acids. The primer binding sites used for the construction of the mutated gB-recombination shuttle vectors are shown in Figure 3.3 (see also Table 3.1). The insertion of either gB-Pert or gB-Gus mutations within the pSacB/RecA-gB plasmid was performed by PCR overlap restriction with subsequent extensions of two individual PCR products by the tagging of the gB-tail mutated sequences with a stop codon and a BamHI site (see Materials and Methods).

Construction of the KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus

The complete KSHV genome was recently cloned into a BAC, yielding BAC36 and enabling the genetic manipulation of the KSHV genome in E. coli. KSHV-BAC36 constitutively expresses the GFP gene, allowing the detection of infected or transfected eukaryotic cells containing KSHV-BAC36 genome. A targeted two-step mutagenesis replacement procedure was employed to construct gB-mutant-KSHV-BAC36 genome, with engineered gB carboxyl tail truncations, in E.coli. The details of the two-step mutagenesis procedure are described in the Materials and Methods. Briefly, gB-mutant homologous recombination shuttle vectors (gB-Pert and gB-Gus) containing the gB
Figure 3.3 Schematic illustration of the insertion of gB-Pert and gB-Gus mutations within the pSacB/RecA-gB plasmid. This model shows the primer binding sites used for the construction of the mutated gB-recombination shuttle vectors: pSacB/RecA-gB-Pert and pSacB/RecA-gB-Gus. The insertion of gB-Pert and gB-Gus mutations within the pSacB/RecA-gB plasmid were performed by PCR overlap extensions of two individual PCR product by the tagging of the gB-tail mutated sequences with a stop codon and a BamHI site. The gB-Pert mutation within the carboxyl tail was created by producing two PCR products which constituted a 5’-end (Primers IX & IV; Table 3.1) and a 3’-End (Primers III & X; Table 3.1). The 5’-End and 3’-End were cut with BamHI and subsequently ligated to each other. A final round of PCR (Primers IX and X; Table 3.1) was performed to amplify the mutated gB-Pert region including the 1 kb upstream arm A and 1 kb downstream arm B sequences. A similar strategy was employed for the insertion of the gB-Gus carboxyl tail mutation: 5’-End (Primers IX & II; Table 3.1) and 3’-End (Primers I & X; Table 3.1).
mutant gene, specifying mutations in the carboxyl tail, along with 1 kb homologous upstream (arm A) and downstream (arm B) flanking sequences were utilized in the first homologous recombination step (Figure 3.4). The first step includes the incorporation of the shuttle vector into the KSHV-BAC36 genome, thus forming a co-integrate. The homologous recombination mechanism is mediated by the RecA gene, which is provided by the recombination shuttle vector. The second recombination step, which is also mediated by the RecA enzyme, allows for the resolution of the co-integrate and restoration of the wild-type KSHV-BAC36 genome or intended KSHV-BAC36-gB-mutant. This resolution step results in complete excision of the shuttle vector from the KSHV-BAC36-SacB/RecA-gB-mut co-integrate, which leaves the targeted mutagenic sequences within the KSHV-BAC36 genome. Since the resolution of the co-integrate does not occur with high frequency, negative selection pressure against the bacteria harboring the cointegrate occurs due to SacB gene expression of levansucrase which converts the supplemented sucrose within the LB-broth and/or plates into levan, a bacterial toxin. In this study, we utilized the two-step SacB/RecA mutagenesis procedure to insert gB-carboxyl tail truncations within the KSHV-BAC36 genome (KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus).

**Genetic Characterization of the KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus**

The KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus constructs were tested for the presence of engineered insertion of the Opal-stop codon and a BamHI site within the carboxyl tail of the gB gene. Restriction enzyme analysis of the wild-type KSHV-BAC36 and mutant KSHV-BAC36-gB-Pert & KSHV-BAC36-gB-Gus with BamHI revealed similar DNA fragmentation patterns, with the exception that a new BamHI
Figure 3.4. A schematic illustration of the two-step SacB/RecA shuttle mutagenesis system for the insertion of point mutations within the KSHV-BAC36 genome. A schematic model of the two-step SacB/RecA mutagenesis system with pPERT and pGUS as shuttle vectors. This vector contains a R6kγ origin, an Amp-resistant gene, RecA for the restoration of homologous recombination competence, and the SacB gene intended to act as a negative selection step. The two BAC modification phases are illustrated: (1.) Shuttle vector pPERT and pGUS are co-integrated into BAC via homologous recombination through ‘A’. (2.) There is a second homologous recombination event in the resolution step of the co-integrant to eliminate the previous vectors and any other unnecessary sequences from the co-integrate. Due to the loss of the SacB gene, the resolved BACs (KSHV/BAC36-gB-Pert and KSHV/BAC36-gB-Gus) were grown on sucrrose and chloramphenicol plates.
(1.)

WT KSHV-BAC36

Co-integrate

Co-integration (Chl+ and Amp+)

(2.)

Resolution (Select for growth on Chlor/Sucrose Plates)

BAC with Mutation

Original BAC
restricted fragment, which appeared in the vicinity of the 9.4 kb lambda molecular marker. The new BamHI DNA fragment present within the KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus (Figure 3.5; Lanes 3 and 4) is noticeably absent in the restricted DNA fragmentation pattern of the wild-type KSHV-BAC36 genome (Figure 3.5; Lane 2). The new BamHI DNA fragment is caused by the insertion of the BamHI site at the carboxyl terminal domain of the gB gene within the KSHV-BAC36 genome. The remaining restriction pattern of the mutant KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus is identical to the KSHV-BAC36 fragment pattern, thus the engineered insertion of the BamHI site with the preceding contiguous opal stop codon was precisely inserted within the KSHV-BAC36 genome without any obvious deletions of large regions of the KSHV-BAC genome.

In order to confirm the precise location of the inserted mutation within the KSHV-BAC, we used a diagnostic PCR assay to ensure the proper orientation of the inserted opal stop codon and BamHI site within the carboxyl tail of the gB gene within the KSHV-BAC36 genome. Primers IX & VI specific for the gB-Gus mutation were used to amplify a diagnostic PCR product ~3kb (Table 3.1 and Figure 3.6; Lane 2). Primer IX, the 5’primer, binds to the 5’-end of homology arm A and primer VI binds to the gB-Gus inserted mutation: opal stop codon and the engineered BamHI site, thus a PCR product would only arise if the inserted mutation is within the precise intended location. Amplification using primers IX and VI on the wild-type KSHV-BAC36 DNA does not produce a PCR product (Figure 3.6; Lane 3). Primers IX & VIII specific for the gB-Pert mutation were used to amplify a diagnostic PCR product ~3kb (Table 3.1 and Figure 3.6; Lane 1). Primer IX binds to the 5’-end of homology arm A and primer VIII
Figure 3.5. Restriction Fragment Analysis with BamHI digestion of wild-type KSHV-BAC36 and mutants KSHV-BAC36-gB-Pert & KSHV-BAC36-gB-Gus.

BamHI digestion of the wild-type KSHV-BAC36 episome (Lane 2) along with BamHI digestion of mutant KSHV-BAC36 episomes: KSHV-BAC36-gB-Pert (Lane 3) and KSHV-BAC36-gB-Gus (Lane 4). Lambda-HindIII DNA digested molecular size markers are shown in Lane 1. BamHI restriction fragment analysis of the gB-carboxyl tail mutants of KSHV-BAC36 in comparison with the wild-type KSHV-BAC36 episome clearly shows size differences within the fragmentation pattern. An approximate 9.4 kb BamHI restricted DNA fragment is clearly present in the lane containing mutant KSHV-BAC36-gB-Pert episome (Lane 3) and slightly larger BamHI restricted DNA fragment is present in the lane containing mutant KSHV-BAC36-gB-Gus episome (Lane 4), yet these bands around the 9.4 kb lambda molecular marker fragment was absent within the lane containing the wild-type KSHV-BAC36 episome (Lane 2). The BamHI enzyme recognized an additional DNA sequence, GGATCC (BamHI) inserted directly after the artificial Opal-stop codon introduced into both gB-mutant KSHV-BAC36 episomes: KSHV-BAC36-gB-Pert (Lane 3) and KSHV-BAC36-gB-Gus (Lane 4). The missing DNA fragment (9,416bp) within the wild-type KSHV-BAC36 genome is demarcated by a yellow star in Lane 2.
binds to the gB-Pert inserted mutation: opal stop codon and the engineered BamHI site, thus a PCR product would only arise if the inserted mutation is within the precise intended location. Amplification using primers IX and VIII on the wild-type KSHV-BAC36 DNA did not lead to the production of a PCR product (not shown). The confirmatory results of the restriction analysis and diagnostic PCRs are consistent with the proper insertion of the gB-Pert and gB-Gus mutations within the gB gene of the KSHV-BAC genome. The KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus constructs were sequenced from the 3’-end of the gB gene (gB’s original stop codon); the sequence results show the precise insertion of the both the Opal stop codon and BamHI site within the carboxyl tail of gB (Figure 3.7).

**Transient Transfection Assay to Determine Virus-Induced Cell Fusion Caused by either KSHV-BAC36-gB-Pert or KSHV-BAC36-gB-Gus**

To assess the fusogenic potential of the mutations within the carboxyl tail domain of KSHV-gB, we developed a transient transfection assay, in which KSHV-BAC eukaryotic transfected cells are induced with TPA for 48 hours and viewed under fluorescence microscopy for cell fusion. Column-purified BAC DNA (wt-KSHV-BAC36, KSHV-BAC36-gB-Pert, and KSHV-BAC36-gB-Gus) were used to transfect 293 cells (Figure 3.8; A1, B1 and C1, respectively). Transfected cells harboring the KSHV-BAC genome constitutively expresses the GFP gene, thus allowing for facile detection of cells containing the KSHV-BAC genomes via fluorescence microscopy. Also, BAC-transfected cells were exposed to TPA (20ng/ml) for 48 hours in order to induce late gene
Figure 3.6. Diagnostic PCR analysis of the viral mutants: KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus. Diagnostic PCR assay was used to confirm the respective gB-carboxyl-tail mutations within the KSHV-BAC36 genome: KSHV-BAC36-gB-Pert (Lane 1) and KSHV-BAC36-gB-Gus (Lane 2). The KSHV-BAC36-gB mutant clones were produced via the modification of KSHV-BAC36 by pGUS and pPERT recombination shuttle vector using a built-in resolution strategy at homology arm ‘A’. The PCR product of the region encompassing the mutated gB-carboxyl-tail region (1kb upstream of the gB start codon to the mutated gB-tail region) using diagnostic primers that only allowed for amplification of a 3kb product from the mutated gB-KSHV-BAC36s (Lanes 1 & 2) and not from wild-type KSHV-BAC36 genome (Lane 3). The 2-log DNA ladder was used as the molecular size marker (Lane 4).
Figure 3.7. Nucleotide sequence analysis of KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus. DNA sequencing was performed of the 3’-ends of the two KSHV-gB-carboxyl-tail recombinant BAC clones after the modification of the gB-carboxyl tail by the insertion of both the Opal-stop codon and BamHI site to produce KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus. The KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus were sequenced using the ABI Prism 310 Genetic Analyzer. The DNA sequence ‘GGATCCTCA’ are highlighted, which includes the BamHI site as well as the Opal-stop codon of gB-Gus and gB-Pert.
expression and enhance virus-induced cell fusion. Wild type-KSHV-BAC36 (Figure 3.8; A1) and mutant KSHV-BAC36-gB-Pert (Figure 3.8; B1) did not produce significant cell fusion, yet KSHV-BAC36-gB-Gus (Figure 3.8; C1) seemed to produce limited amounts of virus-induced cell fusion. The KSHV-BAC36-gB-Gus (Figure 3.9; A1, B1 and C1) was further examined by the transient transfection cell fusion assay and also showed small levels of virus-induced cell fusion.

**DISCUSSION**

Kaposi’s Sarcoma Herpes Virus (KSHV) is considered to be the etiologic agent of Kaposi’s Sarcoma. KSHV relies on different viral membrane proteins for virion attachment to cell surfaces and penetration into cells. Glycoprotein B (gB) is conserved among all human and animal herpesviruses, and it is thought to mediate membrane fusion phenomena during virus entry and virus-induced cell fusion. The role of the gB carboxyl terminus in virus-induced cell fusion was investigated by constructing recombinant KSHV strains using the KSHV genome cloned into a bacterial artificial chromosome (BAC36). Two recombinant BAC36-derived genomes were constructed specifying truncations that fully or partially truncated a predicted α-helical structure of the gB carboxyl terminus known to be involved in virus-induced cell fusion from studies with the herpes simplex virus type 1 (HSV-1) gB. The first specific gB mutation site, gB-Pert, within the carboxyl tail was selected to be identical to a previous KSHV study describing an *in vitro* cell fusion assay by Pertel (Pertel, 2002). This gB mutation caused enhanced cell fusion in the context of an *in vitro* virus-free cell fusion assay, presumably, in part, because deletion of the 58 carboxyl terminal aa of gB containing putative endocytotic motifs resulted in increased accumulation of gB on cell surfaces. The second specific gB
Figure 3.8. Observation of virus-induced cell fusion via transient transfections of KSHVgB Mutant BACs. Transient transfection of 293 cells with wild KSHV-BAC36 DNA (A1), KSHV-BAC36 gB-Pert DNA (B1) and KSHV-BAC36-gB-Gus DNA (C1). The BAC-transfected 293 cells were induced with TPA induction for 48 hours. Wild-type KSHV-BAC36 and KSHV-BAC36-gB-Pert transfected cells produced no significant cell fusion. The KSHV-BAC36-gB-Gus seemed to produced limited amounts of virus-induced cell fusion. The panels show the corresponding cells via fluorescence and phase-contrast microscopy (magnification, X200). Matching phase-contrast images are shown in panels A2, B2 and C2.
Figure 3.9. Observation of cell-to-cell membrane fusion via transient transfections of KSHV-BAC36-gB-Gus DNA. Transient transfection of 293 cells with KSHV-BAC36-gB-Gus DNA (panels A1, B1 and C1). The KSHV-BAC36-gB-Gus transfected 293 cells were induced with TPA for 48 hours. The KSHV-BAC36-gB-Gus seemed to produce limited amounts of cell-to-cell fusion. The panels show the corresponding cells via fluorescence and phase-contrast microscopy (magnification, X400). Matching phase-contrast of KSHV-BAC36-gB-Gus images are shown in panels A2, B2 and C2.
mutation site (gB-Gus) was chosen based on studies with the HSV-1 gB homologue which have suggested that the smaller of the two major α-helical domains in the gB carboxyl terminus, which are conserved between KSHV and HSV-1 gB, is important in virus-induced cell fusion and virus-free cell fusion assays.

Consistent with previous findings with the KSHV-gB virus-free cell fusion assay and HSV-1-gB regulated virus-free cell fusion and virus-induced cell fusion assays, the KSHV-gB mutant genes (expressing carboxyl terminal truncations gB-Pert and gB-Gus), produced higher amounts of fusion than KSHV-BAC36 containing the wild-type-gB. Moreover, the gB-Gus truncation (analogous to the HSV-1 gB truncation), which interrupts the smaller of the two carboxyl-terminal α-helical domains of KSHV-gB, seemed to produce higher amounts of virus-induced cell fusion than the gB-Pert truncation or the wild-type gB. These preliminary results suggest that despite the evolutionary divergence of KSHV gB and HSV-1 gB, functional domains involved in virus-induced cell fusion are conserved suggesting a similar modus operandi. KSHV recombinant viruses carrying gB carboxyl terminal truncations and other single amino acid mutations would be instrumental in assessing the role of the gB carboxyl terminus in virus-induced cell fusion.

One of the main difficulties in quantitatively assessing the extent of virus-induced cell fusion by the different KSHV mutants is that under BAC transfection conditions only a small percentage of the cells are capable of expressing the mutant genes. This limited expression is due to the low levels of BAC-KSHV transfection rates (less than 10% of the cells) and the low reactivation rates of the transfected cells upon induction with phorbol esters (less than 20% of the transfected cells). To better assess the role of KSHV-gB
carboxyl terminal truncations in virus-induced cell fusion, permanent TIME cell lines are being constructed and characterized. These permanently transformed TIME cells (telemorase immortalized microvascular endothelial cells) would serve as useful reservoirs for efficient virus production and infection of cell cultures to adequately quantitate the varying amounts of virus-induced cell fusion. The KSHV-BAC36-gB-mutant-TIME cells could also potentially allow for sufficient mutant virus production for structural and biochemical experiments.

We have demonstrated here for the first time that the KSHV BAC36 genome can be manipulated in E. coli to generate site-directed mutations within a viral gene. The RecA/SacB two-step mutagenesis system implemented in this study has allowed for the rapid generation of mutant KSHV-BAC36 genomes, specifically the potential to deliver point mutations. The KSHV-BAC36-gB-Pert and KSHV-BAC36-gB-Gus viral genomes produced by the two-step bacterial mutagenesis system would serve crucial roles in the delineation of KSHV-gB functional domains involved in membrane fusion in the future.

REFERENCES


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CHAPTER IV

MUTAGENESIS OF THE KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS GENOME BY USING A GET RECOMBINATION STRATEGY

INTRODUCTION

Herpesviruses are important pathogens associated with a wide range of diseases in both humans and animals. Members of the Herpesviridae family possess large DNA genomes (Knipe et al., 2001). Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, belongs in the gammaherpesvirinae subfamily and is believed to be the etiologic agent of the following diseases: 1.) Kaposi’s sarcoma, 2.) primary effusion lymphoma, and 3.) a subset of multicentric Castleman’s disease (Moore and Chang, 2001). The KSHV lifecycle is similar to other gammaherpesviruses, which consist of both latent and lytic phases; however, the fundamental aspects of KSHV infection regarding virology and cell-biology in KSHV-related malignancies remain elusive and poorly understood (Ablashi et al., 2002). KSHV possesses approximately ninety ORFs within its genome (Russo et al., 1996). Many of the individual KSHV genes have been cloned and characterized in cell culture, yet the true function of a herpesviral gene could only be assessed in the context of the viral genome (Moore and Chang, 2001).

 Revolutionary experiments involving the propagation of infectious herpesviral clones as bacterial artificial chromosomes (BACs) have lead to the unparalleled experimental manipulation of herpesviral genomes, which exhibit cell-associated properties and slow replication kinetics. The BAC technology has allowed the application of tools of prokaryotic molecular biology to the study of viral genetics, whereby permitting the genetic analysis of many herpesviruses (Borst et al., 1999; Britt, 2000;
Brune et al., 1999; Delecluse et al., 2001; Janz et al., 2000; Messerle et al., 1997; Smith and Enquist, 1999; Smith and Enquist, 2000; Yu et al., 2000; Zhou et al., 2002).

Recently, a recombinant KSHV, BAC36, has been produced by cloning the full-length KSHV genome into a bacterial artificial chromosome (BAC) and recovering it in 293 cells (Zhou et al., 2002). Recombinant virions generated from BAC36 have been recovered in 293 cells and are highly infectious to target cells, with primary-infection efficiency close to 90-95% (Gao, Deng, and Zhou, 2003). BAC36-KSHV replication in susceptible cells resembles an *in vivo* infection, in which KSHV quickly establishes latent infection following primary infection without active productive lytic replication (Zhou et al., 2002). KSHV, similar to the other viruses in the Herpesvirus Family, specifies numerous glycoproteins which are expressed during the virus lifecycle. Many of these KSHV glycoproteins have been shown to be important in virus entry.

The bacterial GET recombination strategy (Narayanan et al., 1999; Orford et al., 2000) was employed to precisely engineer deletions of glycoproteins within the KSHV-BAC36 genome. This system utilizes the pGET-Rec plasmid for bacterial recombination thus allowing for insertional/deletional mutagenesis of the KSHV-BAC36 genome in *E. coli*. The GET-Rec recombination system involves the coordinate induction with L-arabinose of the plasmid encoding the RecE and RecT genes obtained originally from the Rac bacteriophage, which mediates homologous recombination between linear DNA fragments (homologous chimeric PCR products) and circular episomal DNA (KSHV-BAC36). Linear PCR products are degraded quickly in DH10B cells by the RecBCD exonuclease but this nuclease degradation is inhibited by the gam gene of the lambda bacteriophage provided by the pGET-REC plasmid, which is expressed along with RecE
and RecT genes on the same L-arabinose inducible polycistrionic mRNA. The capability of inducing site-specific recombination in RecA-negative DH10B cells allows for the precise engineering of site-specific deletions of the KSHV-glycoproteins within the KSHV-BAC36 genome. The simultaneous facilitation of the GET- Recombination system to target site-specific mutagenesis of the KSHV-BAC36 and generate a panel of KSHV-BAC36 glycoprotein deletions is extremely useful given the fact that Herpesvirus genes are directionally expressed and the regulatory regions of its genes normally overlap the contiguous upstream or downstream gene sequences. These mutant KSHV-BACs harboring glycoprotein deletions would serve as critical tools to address the molecular dynamics of viral entry mediated by KSHV glycoproteins.

MATERIALS AND METHODS

Cells and Viruses

293 cells were grown in Dulbecco’s modified Eagle medium (DMEM; GIBCO-BRL, Grand Island, N.Y.) supplemented with 2 mM glutamine, 10% fetal calf serum, and antibiotics. KSHV BAC36 contains the green fluorescent protein (GFP) gene cassette under the control of the human cytomegalovirus immediate-early promoter, constitutively expressing the GFP gene, and inserted between KSHV ORF18 and KSHV ORF19 (Zhou et al., 2002).

Construction of a Panel of KSHV-BAC36 mutants with deletions of gB, gH, K8.1, gL and gM

Insertion-deletion mutagenesis of KSHV-BAC36 DNA was accomplished in Escherichia coli using the λgam, recE recT (GET) recombination system (Narayanan et al., 1999; Orford et al., 2000), as described previously for mutagenesis of the KSHV genome (Luna et al., 2004). Electrocompetent KSHV-BAC36 DH10B E. coli cells were
transformed with the plasmid pGETrec, which contains the genes encoding recE, recT and bacteriophage λ gam, grown on plates containing chloramphenicol (12.5µg/ml) and ampicillin (100µg/ml). Individual colonies were picked and grown overnight in Luria-Bertani (LB) medium containing chloramphenicol and ampicillin. The next day, the culture was inoculated into 250ml of LB containing chloramphenicol and ampicillin until a 0.4 optical density was reached at 600nm. Addition of L-arabinose to a final concentration of 0.2% (w/v) and further incubation of 40 minutes induced the expression of the recE, recT and λ gam genes from the plasmid pGETrec. The bacteria cells were then harvested and made electrocompetent. For the gB deletion-kanamycinR insertion mutation within the KSHV-BAC36, a PCR fragment containing the kanamycinR gene cassette flanked by approximately 60bp of gB homologous viral sequences on both sides was used for recombination to construct BAC36ΔgB, containing the kanamycinR gene cassette within the targeted deletion of the majority of the KSHV genomic region. For the gH-KanR mutation, a PCR fragment containing a kanamycinR gene cassette flanked by approximately 60bp of gH homologous viral sequences on both sides was used for recombination to construct BAC36ΔgB, which contained the kanamycinR gene cassette within the targeted deletion of the majority of gH gene within the KSHV genomic region. For the K8.1-KanR mutation, a PCR fragment containing a kanamycinR gene cassette flanked by approximately 60bp of K8.1 homologous viral sequences on both sides was used for recombination to construct BAC36ΔK8.1, which contained the kanamycinR gene cassette within the targeted deletion of the majority of the K8.1 gene within the KSHV genomic region. For the gL-KanR mutation, a PCR fragment containing a kanamycinR gene cassette flanked by approximately 60bp of gL homologous viral sequences on both
sides was used for recombination to construct BAC36∆gL, which contained the kanamycin\(^R\) gene cassette within the targeted deletion of the majority of the gL gene within the KSHV genomic region. For the gM-Kan\(^R\) mutation, a PCR fragment containing a kanamycin\(^R\) gene cassette flanked by approximately 60bp of gM homologous viral sequences on both sides was used for recombination to construct BAC36∆gM, which contained the kanamycin\(^R\) gene cassette within the targeted deletion of the majority of the gM gene within the KSHV genomic region. Briefly, 40\(\mu\)l of electrocompetent DH10B cells, harboring both KSHV-BAC36 and pGETrec, were electroporated with 200ng of each chimeric PCR product, which encoded the kanamycin\(^R\) gene cassette with respective flanking KSHV homologous sequences, to delete the target gene (gB, gH, gpK8.1, gL or gM) using standard electroporation parameters (1.8kV/cm, 200\(\Omega\) and 25\(\mu\)F). Following electroporation, cells were grown in 1ml of LB for 60 min and subsequently streaked onto LB agar plates containing both chloramphenicol (12.5 \(\mu\)g/ml) and kanamycin (50\(\mu\)g/ml). Mutant KSHV-BAC36 DNAs, containing a glycoprotein deletion in either the gB, gH, gL, gpK8.1, gL or gM gene, was isolated from bacterial colonies and a second round of electroporation was performed to remove the plasmid pGETrec. Following electroporation, cells were grown on agar plates containing both chloramphenicol and kanamycin.

**Confirmation of the Glycoprotein Deletion within the KSHV-BAC36 Genome**

KSHV BAC DNAs (BAC36, BAC36∆gB, BAC36∆gH, BAC36∆K8.1, BAC36∆gL and BAC36∆gM) were purified from 1 liter of BAC cultures by using a large-construct kit (Qiagen, Valencia, Calif.). BAC DNA was digested with KpnI and run on 0.8% agarose gels, and the restricted DNA was transferred to charged nylon
membranes (Bio-Rad, Richmond, Calif.). Southern blot hybridization was performed with a biotin-labeled kanamycin resistance gene probe by labeling a 1.1-kb kanamycin PCR fragment with biotin (New England Biolabs, Boston, Mass.). Chemiluminescence detection of the DNA was performed by using a North2South chemiluminescence hybridization and detection kit as described by the manufacturer (Pierce Inc., Rockford, Ill.).

**Stable Transfection of Mutant KSHV-BAC DNAs**

Transient transfection of 293 cells with the respective members of the panel of KSHV-BAC36 mutant DNAs was performed by using Superfect (Qiagen). 293 cells were grown to 80% confluence in six-well plates. Cells were transfected with KSHV-BAC36 mutant DNA mixed with Superfect in DMEM as recommended by the manufacturer. After 4 h of incubation at 37°C, the medium was removed from the transfected 293 cells, and the cells were washed with PBS. One day after transfection, fresh DMEM with 10% fetal calf serum and hygromycin B (100 µg/ml) was added. Cells were fed weekly with fresh DMEM with the same concentration of hygromycin B. After 6 weeks, hygromycin B resistant colonies were picked, expanded and subsequently frozen in liquid nitrogen for later use.

**RESULTS**

**Construction of a Panel of Mutant KSHV-BAC36**

The KSHV genome encodes several glycoproteins, gB, gH, gL and gM, which possess conserved sequence homology among all herpesvirus subfamilies; in addition, the K8.1 glycoprotein, which does not possess conserved sequence homology with any
### TABLE 4.1. Synthetic oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
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<tr>
<td>5'-gB-KanF</td>
<td>ACCAAACCTGGTGAGGAAGCATCTGTCTCCTAAAGAGTGCTGGACTTTT TACCAGTTTCAGATAGCCACCTTGGTGCTCTCAAATCTCTGATGTTA</td>
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<tr>
<td>3'-gB-KanR</td>
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<tr>
<td>5'-K8.1-KanF</td>
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<tr>
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other herpesvirus glycoproteins, exhibits positional homology with two EBV glycoproteins: gp350/220 and gp42/38. The KSHV genome has been successfully cloned into a bacterial artificial chromosome, named KSHV-BAC36, thus enhancing the feasibility of KSHV mutagenesis, particularly via the implementation of bacterial recombination mechanisms. The bacteria based GET homologous recombination system was employed to construct a KSHV-BAC36 panel of mutants in \textit{E. coli} depicted in Figure 4.1 The panel of KSHV mutants is listed as follows: KSHV-BAC36\(\Delta\)gB, KSHV-BAC36\(\Delta\)gH, KSHV-BAC36\(\Delta\)K8.1, KSHV-BAC36\(\Delta\)gL and KSHV-BAC36\(\Delta\)gM (Sequence of Primers used for recombination and diagnostic assays are shown in Table 4.1).

**Genetic Characterization of Glycoprotein-Deleted Mutant KSHV-BAC36 DNAs**

Qiagen purified glycoprotein-deleted mutant KSHV-BAC36 DNA was isolated from bacteria and used in a diagnostic PCR to determine the presence of the kanamycin\(^R\)-insertion/glycoprotein-deletion mutation within the targeted region within the KSHV genome. \(5'\)gB-Zt and \(3'\)gB-nostop was designed to amplify the entire gB gene which approximates to 2.5kb within the wild-type genomes of KSHV-BAC36 episomes or KSHV obtained from induced BCBL-1 cells (Figure 4.2; lanes 1 & 2). The insertion of the kanamycin\(^R\) gene cassette along with the concomitant deletion of a major region of the gB gene is predicted to lead to a PCR product \(\sim\)1.7kb (Figure 4.2; lane 3). \(5'\)gHup and \(3'\)gHdown was designed to amplify the entire gH gene which approximates to 2.3kb within the wild-type genomes of KSHV-BAC36 episomes or KSHV obtained from induced BCBL-1 cells (Figure 4.2; lanes 5 & 6). The insertion of the kanamycin\(^R\) gene cassette along with the concomitant deletion of a major region of the gH gene is predicted
**Figure 4.1. Schematic illustration of the GET homologous recombination system used on KSHV-BAC36.** This model depicts the deletion of the gB gene via the GET recombination system, the same approach was used to construct the following glycoprotein deletion, which were not illustrated: KSHV-BAC36ΔgH, KSHV-BAC36ΔK8.1, KSHV-BAC36ΔgL, KSHV-BAC36ΔgM. In this illustration detailing the overall recombination strategy used to produce KSHV-BAC36ΔgB, a chimeric PCR fragment containing the kanamycin resistance gene flanked by 60 base pairs of gB homologous sequences was used to construct BAC36ΔgB genome recombinants containing the kanamycin resistance gene cassette within the targeted gB ORF within the KSHV genome.
Construction of gB-KO-BAC

Diagram showing the construction of gB-KO-BAC, with KSHV, BAC36, and a chimeric PCR-product.
to lead to a PCR product ~1.8kb (Figure 4.2; lane 7). 5’K8.1up and F:3’K8.1nostop were designed to amplify the entire K8.1 gene along with the 5’-upstream region which approximates to 1kb within the wild-type genome of KSHV-BAC36 episomes (Figure 4.2; lane 9). The insertion of the kanamycinR gene cassette along with the concomitant deletion of a major region of the K8.1 gene is predicted to lead to a PCR product ~1.4kb (Figure 4.2; lane 10). 5’gL and H: 3’gL were designed to amplify the entire gL gene which approximates to 0.6 kb within the wild-type genomes of KSHV-BAC36 episomes or KSHV obtained from induced BCBL-1 cells (Figure 4.2; lane 11). The insertion of the kanamycinR gene cassette along with the concomitant deletion of a major region of the gL gene is predicted to lead to a PCR product ~1.4kb (Figure 4.2; lane 12). 5’gM-Z and 3’gM-stop was designed to amplify the entire gM gene which approximates to 1.3 kb within the wild-type genomes of KSHV-BAC36 episomes (Figure 4.2; lane 14). The insertion of the kanamycinR gene cassette along with the concomitant deletion of a major region of the gM gene is predicted to lead to a PCR product ~1.6kb (Figure 4.2; lane 15).

Further confirmation of the genetic content of the panel of KSHV-BAC36 mutants was obtained by restriction endonuclease fragment analysis and Southern blotting (Figures 4.3 and 4.4) Restriction enzyme analysis of the panel of KSHV-BAC36 mutants (KSHV-BAC36ΔgB, KSHV-BAC36ΔgH, KSHV-BAC36ΔK8.1, KSHV-BAC36ΔgL and KSHV-BAC36ΔgM) with KpnI revealed similar but noticeably different DNA fragmentation patterns. Restriction enzyme analysis is an important assay when using BACs, maintenance of a restriction pattern ensures that the BAC is still intact and that major deletions of the BAC genome have not occurred, particularly after a mutagenesis procedure. Upon comparison to KSHV-BAC36 (Figure 4.3; Lane 2),
Figure 4.2. Diagnostic PCR analysis of the panel of KSHV-BAC36 mutants.

PCR assay to confirm respective deletions of the glycoprotein genes within the panel of KSHV-BAC36 mutants: KSHV-BAC36ΔgB, KSHV-BAC36ΔgH, KSHV-BAC36ΔK8.1, KSHV-BAC36ΔgL, KSHV-BAC36ΔgM. Amplification of the various glycoprotein regions from wild-type genomes of KSHV-BAC36 and BCBL-1 cells compared to the KSHV-BAC36 mutants clearly showed appropriate size differences between each respective insertional/deletional mutagenesis performed to obtain individual KSHV-BAC36 glycoprotein-deleted mutants. The lambda molecular size markers are shown on Lanes 4, 8, 13. PCR amplification of the wild-type genomes were performed and run in the following lanes: BCBL-1 (Lanes 1 & 5) and BAC36 (Lanes 2, 6, 9, 11 & 14). Primers used for amplification of each glycoprotein region are listed in table 4.1.
restricted DNA fragments were absent from KSHV-BAC36ΔgH (~3.0kb), KSHV-BAC36ΔK8.1 (4.6kb), KSHV-BAC36ΔgL (2.3kb) (Figure 4.3; Lanes 4, 5 and 6, respectively). KSHV-BAC36ΔgB and KSHV-BAC36ΔgM (Figure 4.3; Lanes 3 and 7, respectively) show remarkably similar patterns of restriction when compared to BAC36, which is due to the location of the kanamycin insertion, which are located on the larger restricted DNA fragments thus the size differential is beneath the sensitivity of the KpnI fragment analysis. These results show that the panel of KSHV-BAC mutants (KSHV-BAC36ΔgB, KSHV-BAC36ΔgH, KSHV-BAC36ΔK8.1, KSHV-BAC36ΔgL and KSHV-BAC36ΔgM) maintained their genome after the GET-REC mutagenesis procedure.

To confirm the presence of only one insertion of the kanamycin resistance cassette within the KSHV-BAC genome due to the GET-REC mutagenesis system, Southern blotting with a biotinylated kanamycin resistance gene probe for the KpnI digested panel of KSHV-BAC36 mutants revealed the presence of a unique kanamycin resistance cassette insertion within each KSHV-BAC mutant’s KpnI-restricted DNA fragment pattern (Figure 4.4; Lanes 3-7). The varying sizes of hybridized fragments indicate the different locations of the kanamycin insertion of the KSHV genome consistent with the differing positions of the glycoproteins on the viral genome. The KSHV-BAC36 genome was used as a negative control; the biotinylated kanamycin probe did not hybridize with the KSHV-BAC36 genome (Figure 4.4; Lanes 2). A prebiotinylated molecular weight ladder was used for DNA fragment size determination (Figure 4.4; Lane 1). These results are consistent with the insertion of the kanamycin
Figure 4.3. Restriction Fragment Analysis with \textit{KpnI} digestion of KSHV-BAC36 and the panel of KSHV-BAC36 mutants. \textit{KpnI} digestion of the wild-type KSHV-BAC36 episome (Lane 2) along with \textit{KpnI} digestion of the panel of KSHV-BAC36 mutant episomes: KSHV-BAC36ΔgB (Lane 3), KSHV-BAC36ΔgH (Lane 4), KSHV-BAC36ΔK8.1 (Lane 5), KSHV-BAC36ΔgL (Lane 6), KSHV-BAC36ΔgM (Lane 7). Molecular size markers are shown in Lane 1. Restriction fragment analysis of the panel of KSHV-BAC36 mutants in comparison with wild-type genomes of KSHV-BAC36 clearly shows size differences within the KSHV-BAC36ΔgH (Lane 4), KSHV-BAC36ΔK8.1 (Lane 5) and KSHV-BAC36ΔgL (Lane 6) when compared to the wild-type KSHV-BAC36 episome (Lane 2). Bands that were present within the wild-type KSHV-BAC36 episome (Lane 2) but are missing due to alterations caused by the insertion of the kanamycin resistance cassette within the KSHV-BAC36 genome are demarcated by yellow arrows in Lanes 3, 4 and 5.
resistance gene cassette within the targeted glycoprotein-deletions within the panel of KSHV-BAC36 mutants.

Since the confirmation of the panel of mutant KSHV-BAC episomes was established, stable 293 cell lines of the panel of mutant KSHV-BAC36 episomes (293-KSHV-BAC36ΔgB, 293-KSHV-BAC36ΔgH, 293-KSHV-BAC36ΔK8.1, 293-KSHV-BAC36ΔgL and 293-KSHV-BAC36ΔgM) were produced via hygromycin selection over a period of six weeks (Figure 4.5; Panels A-E). These established cell lines harbor mutant KSHV-BAC episomes, and the GFP gene under the eukaryotic CMV promoter allow for detection of the stable cells maintaining the mutant KSHV-BACs via fluorescence microscopy. This study has utilized the GET-Rec recombination system in E.coli to effectively target the KSHV glycoproteins within the KSHV genome for deletional/insertional mutagenesis procedures (Figure 4.6).

**DISCUSSION**

Characterization of KSHV has relied on the growth of the virus in cell culture systems (Moore and Chang, 2001). The isolation and characterization of cell lines of pleural effusion lymphomas (PELs) has allowed the culturing of KSHV, wherein KSHV is primarily maintained in latent replication but can be reactivated into lytic replication through induction with various chemicals, such as phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate (TPA) (Arvanitakis et al., 1996; Cannon et al., 2000; Carbone et al., 2000; Carbone et al., 1998; Cesarman et al., 1995; Drexler et al., 1998; Gao et al., 1996b; Gao et al., 1999; Katano et al., 1999; Lacoste et al., 2000; Renne et al., 1996; Uphoff et al., 1998).

The ability to grow KSHV in PEL cell lines has been vital in the development of
Figure 4.4. Southern Blot Analysis with a kanamycin probe of the panel of KSHV-BAC36 mutants. The KpnI restriction pattern from Figure 2.3 was hybridized with a probe derived from a kanamycin PCR product that was labeled with biotin. Biotinylated molecular size markers are shown in Lane 1. The biotinylated kanamycin probe did not hybridize with KSHV-BAC36 (Lane 2), which does not possess a kanamycin cassette; however, the probe hybridized precisely at one location within the KSHV-BAC36 mutant episomes (Lanes 3-7), which indicates that there is only one copy of the kanamycin cassette per KSHV-BAC36 mutant episome: KSHV-BAC36ΔgB (Lane 3), KSHV-BAC36ΔgH (Lane 4), KSHV-BAC36ΔK8.1 (Lane 5), KSHV-BAC36ΔgL (Lane 6), KSHV-BAC36ΔgM (Lane 7). The biotinylated kanamycin probe hybridized to differing KpnI restriction fragments within the respective KSHV-BAC36 mutants, which is in accordance with the differing locations of the glycoprotein genes along the KSHV genome.
Figure 4.5. Stable 293 cell lines harboring Mutant-KSHV-BAC36 episomes. 293 cells were transfected with the respective mutant KSHV-BAC36 DNAs and subsequently selected with hygromycin over a period of six weeks. The 293-KSHV cell lines were produced and labeled in the following panels: A.) 293-KSHV-BAC36ΔgB, B.) 293-KSHV-BAC36ΔgH, C.) 293-KSHV-BAC36ΔK8.1, D.) 293-KSHV-BAC36ΔgL, E.) 293-KSHV-BAC36ΔgM. The panels show the corresponding cells via fluorescent microscopy (magnification, X200).
Figure 4.6. Overall strategy leading to the generation of recombinant KSHV-BAC36ΔgB with the subsequent production of hygromycin resistant 293 cells harboring the KSHV-BAC36ΔgB. This model depicts five steps required for the production of hygromycin resistant 293 cells harboring the KSHV-BAC36ΔgB genome, the same approach was used to produce hygromycin resistant 293 cells harboring the following glycoprotein deletions within the KSHV-BAC36: KSHV-BAC36ΔgH, KSHV-BAC36ΔK8.1, KSHV-BAC36ΔgL, KSHV-BAC36ΔgM (not shown). Step 1, Electrocompetent E. coli DH10B cells harboring the KSHV-BAC36 genome were transformed with plasmid pGETrec. Step 2, Transformation of E. coli DH10B-KSHV-BAC36 with a linearized chimeric PCR product containing the entire Kanamycin resistance cassette with flanking gB homologous sequences (60 base pairs) thus allowing for most of the gB gene to be deleted and replaced with the kanamycin resistance cassette upon homologous recombination, Step 3. After allowing for homologous recombination in E. coli, clones harboring the KSHV-BAC36 genome were selected for chloramphenicol and kanamycin resistance. Step 4, A second round of electroporation was performed in order to remove the plasmid pGETrec, the recombinant clones harboring the mutant KSHV-BAC36 underwent alkaline lysis and the prepped episomes, containing KSHV-BAC36 ΔgB DNA and pGETrec, were then transformed into E. coli DH10B cells and grown on agar plates containing both chloramphenicol and kanamycin. Step 5, Eucaryotic 293 cells were transfected with KSHV-BAC36ΔgB DNA and selected with hygromycin B.
1. Transformation
   pGETrec → BAC36
   E. coli DH10B

2. Transformation
   BAC36 → pGETrec
   Kan
   E. coli DH10B

3. Homologous Recombination
   BAC36
   Kan

4. Transformation
   mBAC36
   Kan-resistant E. coli DH10B

5. Transfection
   mBAC36
   Kan
   Hyg-resistant 293 cells

Characterizations
KSHV-BAC36
PCR primers

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serologic assays (Gao et al., 1996a; Gao et al., 1996b; Kedes et al., 1996; Simpson et al., 1996) and studies investigating the molecular properties of the virus or individual viral genes in the absence of the context of the virus (Moore and Chang, 2001). In order to fully understand the dynamic interplay of viral and cellular mechanisms, especially during viral entry into susceptible cells, requires the establishment of a reliable primary infection system whereby KSHV mutants can be generated and efficiently produced.

Characterization of viral gene function via the generation of herpesvirus mutants and observation of the resultant phenotypic changes has been critical for understanding the molecular aspects of herpesvirus replication and pathogenesis. In classical herpesviral genetics, recombinant herpesviruses can be generated by homologous recombination in infected eukaryotic cells with large DNA fragments (~1-3kb) specifying homologous viral sequences, which can recombine with shuttle vectors to deliver the engineered mutation into the herpesviral genome (Knipe et al., 2001). Mutagenic approaches based on homologous recombination in eukaryotic cells have been limited to genetic manipulation of only the most rapidly replicating and promiscuous herpesviruses, such as herpes simplex virus (HSV) (Knipe et al., 2001) and pseudorabies virus (PRV)(Post and Roizman, 1981), largely, due to the need of isolating single plaque-forming virions. In contrast, the generation of a homogenous population of KSHV mutants has been untenable due to the difficulties associated with viral propagation and plaque formation, thus severely impeding the systematic delineation of viral gene functions in the context of the viral genome.

Recently, the KSHV genome was cloned into a bacterial artificial chromosome (BAC) and shown to produce infectious virus; recombinant KSHV genome was named
KSHV-BAC36 (Zhou et al., 2002). The KSHV-BAC36 genome allows the genetic manipulation, particularly gene deletions or site-directed mutagenesis, of the KSHV genome using the prokaryotic recombination machinery. Since KSHV is maintained in bacteria, essential genes required for virion morphogenesis can be genetically altered and the mutant viral genome can be recovered as homogeneous population by propagation in bacteria.

This study reported the generation of a panel of mutant-KSHV-BAC36-based genomes, which contain different glycoprotein-deletions. These gene deletions were produced through the use of the pGET-Rec bacterial recombination system for insertional/deletional mutagenesis of the KSHV-BAC36 genome. Targeted deletion of the complete ORFs of herpesviruses for functional analysis of individual genes is complicated by the presence of overlapping ORFs and regulatory sequence elements controlling the gene expression of neighboring genes. The approximate 60bp homology arms used for recombination in this study allowed the deletion of the majority of the targeted gene sequences the glycoprotein intended for deletion with minimal affect on the neighboring genes.

The panel of KSHV-BAC36 mutants (KSHV-BAC36ΔgB, KSHV-BAC36ΔgH, KSHV-BAC36ΔK8.1, KSHV-BAC36ΔgL and KSHV-BAC36ΔgM) as well as other KSHV mutant viruses constructed in this laboratory will be instrumental in determining the role of KSHV-glycoproteins in virus attachment, entry, virion maturation and egress. Furthermore, the panel of stable 293 cell lines harboring the mutant KSHV genomes (293-KSHV-BAC36ΔgB, 293-KSHV-BAC36ΔgH, 293-KSHV-BAC36ΔK8.1, 293-KSHV-BAC36ΔgL and 293-KSHV-BAC36ΔgM) would be useful in the characterization
of these mutant viruses. Unlike transient transfections, these cell lines can produce large amounts of virions for virion structural analysis as well as high MOI- infection of permissive cells.

REFERENCES


CHAPTER V
CONCLUDING REMARKS

SUMMARY

Infectious KSHV inoculum obtained from pleural effusion lymphoma cell lines (PEL) and occasionally from KS tumors have been shown to infect several cell types, including the following cell lines: monocytes, fibroblasts, lymphocytes, and keratinocytes (Cerimele et al., 2001; Foreman et al., 1997; Kliche et al., 2001; Mesri et al., 1996; Moore et al., 1996; Panyutich, Said, and Miles, 1998; Renne et al., 1998; Vieira et al., 2001). Unfortunately, KSHV primary-infection efficiencies in these cells are typically low, and the cultures are unable to efficiently passage the virus or produce large amounts of virus-stock production upon the induction of the infected cell with the phorbol ester, TPA.

The KSHV viral system remains a difficult experimental system due to multiple difficulties associated with virus replication including: the inability to sufficiently passage KSHV; the low primary infection efficiencies; the production and characterization of viral mutants in order to assess the structure and function of individual viral gene products; the low percentage of latent cells that can be reactivated to express lytic genes: the low percentage of cell expressing lytic genes that can actually (Ciufio et al., 2001; Moses et al., 1999).

Recently, the full-length KSHV genome has been cloned into a bacterial artificial chromosome (BAC) and successfully recovered in 293 cells. Wild-type KSHV generated from the BAC clone was named BAC36 and has been shown to be highly infectious to
293 cells and endothelial cells, with primary-infection efficiency close to 90-95% (Gao, Deng, and Zhou, 2003). The work included in this dissertation has focused on the genetic manipulation of the KSHV-BAC36 in order to address the role of K8.1 in viral entry and the role of the gB-carboxyl tail α-helices in virus-induced cell fusion. In addition, a panel of KSHV-glycoprotein mutants was constructed in order to address the specific role of each glycoprotein in viral entry, egress and virus-induced cell fusion.

These investigations have shown that the K8.1 glycoprotein is dispensable for viral entry. The K8.1 glycoprotein is a structural component of the KSHV particle and is thought to facilitate virus entry by binding to heparan sulfate moieties on cell surfaces. To further address the role of the K8.1 glycoprotein in virus infectivity, a K8.1-null recombinant virus (BAC36ΔK8.1) was constructed by deletion of most of the K8.1 open reading frame and insertion of a kanamycin resistance gene cassette within the K8.1 gene. Southern blotting and diagnostic PCR confirmed the presence of the engineered K8.1 gene deletion. Transfection of the wild-type genome (BAC36) and mutant genome (BAC36ΔK8.1) DNAs into 293 cells in the presence or absence of the complementing plasmid (pCDNAK8.1A), transiently expressing the K8.1A gene, produced infectious virions in the supernatants of transfected cells. Hence, these results clearly demonstrated that the K8.1 glycoprotein is not required for KSHV entry into 293 cells.

The role of the gB carboxyl terminus in virus-induced cell fusion was investigated by constructing recombinant KSHV strains using the KSHV genome cloned into a bacterial artificial chromosome (BAC36). KSHV glycoprotein B (gB) is conserved among all human and animal herpesviruses, and it is thought to mediate membrane fusion phenomena during virus entry and virus-induced cell fusion. Two recombinant BAC36-
derived genomes were constructed specifying truncations that fully or partially truncated a predicted alpha helical structure of the gB carboxyl terminus known to be involved in virus-induced cell fusion from studies with the herpes simplex virus type 1 (HSV-1) gB. Mutated gB genes specifying the two gB truncations were introduced via homologous recombination into the KSHV BAC36 viral genome using a shuttle vector containing the mutated gB and several other genes to aid in the selection of the mutant KSHV BACs in *E. coli*. Co-integrates were resolved using SacB as a negative selection marker. gB-carboxyl-tail KSHV-Mutant BACs were transfected into 293 cells and viral lytic replication was induced by TPA. Initial experiments suggested that disruption of the predicted α-helical structure gBtH2 enhanced virus-induced cell fusion. The SacB/RecA two-step mutagenesis system allows for the rapid generation of mutant KSHVs, and this mutagenesis procedure will be useful in the delineation of gB functional domains involved in membrane fusion. Currently, permanently transformed TIME cells are being constructed to better assess the role of the gB carboxyl terminus on virus-induced cell fusion.

The culmination of this dissertation has led to the generation of a panel of mutant-KSHV-BAC36 viral genomes, which specify glycoprotein-deletions within the KSHV genome. Utilization of the pGET-Rec bacterial recombination system for insertional/deletional mutagenesis of the KSHV-BAC36 genome was successfully performed to produce the following KSHV-BAC36 mutants: KSHV-BAC36ΔgB, KSHV-BAC36ΔgH, KSHV-BAC36ΔK8.1, KSHV-BAC36ΔgL and KSHV-BAC36ΔgM. Subsequently, a panel of stable 293 cell lines, harboring the mutant KSHV-BAC genomes, were established: 293-KSHV-BAC36ΔgB, 293-KSHV-BAC36ΔgH, 293-
KSHV-BAC36ΔK8.1, 293-KSHV-BAC36ΔgL and 293-KSHV-BAC36ΔgM. These stable cell lines would serve critical roles in the characterization of these mutant viruses via the production of large amounts of mutant virus stocks and the assessment of the individual glycoprotein role in virus-induced cell fusion.

In conclusion, this dissertation work has capitalized on the recent development of the KSHV-BAC36 genome in order to deliver targeted mutations to the KSHV genome. The results in this dissertation include the first characterization of a KSHV mutant. The results of these investigations have also produced the first targeted point mutations within the KSHV genome. Currently, the work produced herein this dissertation has contributed to the advancement of the genetics and functional characterization of KSHV mutants, and has investigated the function of genes in the context of the viral genome. This work has contributed to the field of KSHV via the elucidation of the structure and function of KSHV-glycoprotein K8.1 and the preparations of a panel of KSHV glycoprotein mutants which would aid in granting insight into the multi-functional roles of glycoproteins throughout the viral lifecycle.

**FUTURE RESEARCH CHALLENGES**

The dynamic interplay between KSHV and the infected cell which ultimately leads to cellular transformation and the subsequent manifestation of malignant tumors and pleural effusion lymphomas, termed as Kaposi’s Sarcoma and Body Cavity Based Lymphomas respectively, is of tremendous importance to the field of viral pathogenesis and oncogenesis, thus research in this area could possibly lead to the elucidation of fundamental mechanisms involved in viral transformation and cancer development. KSHV or human herpesvirus 8 is the most recently identified human herpesvirus, and the
potential cellular pathways and usurp of cellular pathways by the virus has led to intense research effort in this field. The holy grail of this virus would be the elucidation of the viral mechanisms leading to the transformation of the infected cell. The role of glycoproteins in cellular transformation is an interesting supposition; however, the immediate concern regarding glycoproteins is to define the functional characteristics of KSHV glycoproteins in viral entry, egress and virus-induced cell fusion.

These investigations clearly showed the dispensable role of the K8.1-glycoprotein in viral entry. This research supports the model that the K8.1-glycoprotein possesses a functional role in viral egress, since the complementation of the mutant KSHV-BAC36ΔK8.1 with a K8.1-plasmid has led to enhanced viral production from transfected cells when compared to cells transfected with only the BAC36ΔK8.1. This observation is an interesting one in light of recent results presented at the 2004 Kaposi’s Sarcoma-associated herpesvirus workshop, in which a group showed that the complementation of a KSHV-BAC36ΔgB with a gB plasmid corrected an egress defect of cells that were only transfected with the KSHV-BAC36ΔgB genome. gB and K8.1 glycoproteins both bind to heparan-sulfate receptors during the viral entry process, thus gB and K8.1 may also both possess functional roles in viral egress. Herpesviruses glycoproteins have been known to possess redundant functional roles, yet the functional redundancy of glycoproteins varies depending on the individual herpesvirus and these mechanisms merits further investigation.

These studies have also led to construction of gB-mutant KSHVs, specifically with insertion of the opal stop codons within the carboxyl tail of the gB molecule, thus truncating the protein in order to assess the role of the KSHV-gB tail in virus-induced
fusion. Recently another research group has shown that the telomerase-immortalized microvascular endothelial (TIME) permanent cell line is susceptible to high levels of primary infection with KSHV and sustains several passages of the virus, thus stable transfection of TIME cells with mutant KSHV-BACs could theoretically serve as reservoirs for viral production and assist characterization studies of the KSHV-mutant-BACs (Lagunoff et al., 2002). Current experiments are focused on the establishment of permanent TIME cells allowing for an infectious model in which passage of the KSHV-mutant virus would be possible along with the ability to efficiently quantitate virus-induced cell fusion. In addition, permanent TIME cells, harboring members of the panel of KSHV-glycoprotein deletion mutants, are currently being prepared in order to produce an infectious system in which passage of the KSHV mutant virions would be made possible. Once the KSHV-glycoprotein deletion mutant-TIME cells are produced, complementation studies involving transient delivery of the complementing gene could be used to assess the dispensable roles of these glycoproteins in viral entry, egress and virus-induced cell fusion. The complementation system would allow the molecular dissection of functional domains of each glycoprotein and their respective roles in the virus lifecycle. The most appealing experiments would be investigation of the role of glycoproteins in viral transformation of the infected cell. In this regard, the KSHV-glycoprotein deletion mutant-TIME cells would allow us to glean significant findings.

REFERENCES


APPENDIX

LETTER OF PERMISSION TO INCLUDE PUBLISHED WORK

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VITA

Rafael Luna was born in Washington, DC at the Georgetown University Hospital on October 11, 1971, to Rafael and Crescencia Luna. Rafael received his high school diploma from Cardozo Senior High School. In High School he excelled in the competitive swimming arena, he was awarded the Most Outstanding Swimmer in the District of Columbia Public High School System along with several other swimming awards. Rafael attended Southern University in order to obtain his undergraduate degree in Biological Sciences and subsequently graduated in the Fall of 1995. He spent approximately two years in a cardiovascular pathology laboratory at the National Institutes of Health, Heart, Lung and Blood Institute under the tutelage of the late Victor Ferrans, MD., PhD., Chief of the Ultrastructural Pathology Section at NHLBI. Upon graduation, Rafael has accepted a post-doctoral research position investigating the molecular mechanisms of neuronal and cardiovascular stroke at the Morehouse School of Medicine in Atlanta, GA. He has been selected as a Society for Neuroscience Post-doctoral Scholar (2004-2006) and has recently been awarded a Society for Neuroscience-3-year post-doctoral travel fellowship. His hobbies include reading history books and playing soccer every Sunday evening with his major professor and friend, Konstantín G. Kousoulas, Ph.D.