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MOLECULAR DETERMINANTS OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS TUMORIGENICITY

A Thesis

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
Master of Science

In

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Pathobiological
Sciences

By
Haixia Kong
DVM, Nanjing Agricultural University, 2004
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ABSTRACT

A conditional silencing system using anti-gB siRNAs was devised to investigate the structure and function of the KSHV gB. Transient co-transfection of plasmids constitutively expressing gB and anti-gB siRNAs in 293 cells substantially inhibited gB mRNA levels and protein production. Similarly, transient expression of siRNAs into the basal cavity-based lymphoma cells (BCBL-1) caused substantial reduction of gB transcription and protein synthesis. TaqMan real-time PCR and infectivity assays showed that gB was essential for virion egress and infectivity. Transfection of a codon-optimized gB not recognized by the anti-gB siRNAs, efficiently rescued virion egress and infectivity in BCBL-1 cells in the presence of siRNAs inhibiting wild-type gB expression. Virion egress experiments with truncated gBs revealed that removal of the entire predicted cytoplasmic domain of gB increased virion egress suggesting the presence of a egress-regulation domain located proximal to the intramembrane sequence within the cytoplasmic domain of gB. All supernatant virions were infectious on 293 cells indicating that the carboxyl terminus of gB is not essential for either virion egress or virus infectivity. To investigate the potential role of gB in tumorigenesis, BCBL-1 cells transiently transfected with anti-gB siRNAs and codon optimized gB were mixed with Matrigel and injected subcutaneously in nude mice. Direct measurement of tumors revealed that BCBL-1 cells transfected with anti-gB siRNAs produced tumors significantly smaller than mock-transfected BCBL-1 cells. Co-transfection of codon optimized gB appeared to abrogate the inhibition of tumor formation by siRNAs. These results show that gB is important for infectivity, virion egress and pleural effusion lymphoma (PEL) formation in mice. Current work focuses on the use of a lentiviral expression system to generate BCBL-1 cells that constitutively express anti-gB siRNAs to improve inhibition of endogenous gB expression.

CHAPTER I

INTRODUCTION

STATEMENT OF RESEARCH PROBLEMS AND HYPOTHESIS

Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus 8 (HHV-8) is a member of the gamma-2-herpesvirus family (genus Rhadinovirus). In 1994, the virus was first discovered in Kaposi's sarcoma (KS) tumors of AIDS patients. KSHV is associated with Kaposi's sarcoma, primary effusion lymphoma (PEL) or body cavity-based lymphoma (BCBL), and multicentric Castleman's disease. To date, KSHV has shown to be required for disease manifestation, yet certain basic mechanisms by which KSHV promotes oncogenesis and tumorigenicity have not been clearly understood, including the roles of KSHV glycoproteins in PEL.

PEL cell lines represent the most consistent model for the study of KSHV. Most PEL cells are latently infected, and only a very small percentage of infected cells undergo random lytic infection. KSHV encodes a number of glycoproteins, some of which are conserved within the Herpesviridae Family. Glycoprotein B mediates the initial virion binding to glycosaminoglycans, e.g., adherent target cell surface heparan sulfate molecules (Akula et al., 2001b). KSHV gB also binds to $\alpha5\beta3$ integrin through the RGD (Arg-Gly-Asp) motif and its associated signaling pathways that are important for KSHV entry into target cells (Akula et al., 2001a; Birkmann et al., 2001; Garrigues et al., 2008; Wang et al., 2001a). Previous studies from our laboratory showed that the carboxyl terminus of herpes simplex virus type 1 (HSV-1) gB is associated with virus-induced and virus-free cell fusion (Baghian et al., 1993; Foster et al., 2001). Truncation of the carboxyl terminus of pseudorabies virus gB has shown that internalization of gB is not required for gB incorporation into virions nor for its function in either

entry or cell-to-cell spread (Nixdorf et al., 2000; Pertel et al., 2002). It has been reported that gB activates the vascular endothelial growth factor receptor 3 (VEGFR-3) indicating the importance of KSHV in PEL pathogenesis (Zhang et al., 2005).

The overall experimental approach focused on the role of KSHV gB in viral egress and tumorigenicity. Two gB carboxyl terminal truncations having 25 and 56 amino acids deleted were generated. In addition, two other gB deletions lacked the entire cytoplasmic domain of gB and the cytoplasmic domain with part of the carboxyl terminal-most intramembrane sequence. These gB truncations were tested for their ability to rescue siRNA-inhibited gB synthesis with regard to virion egress and infectivity. It was found that the carboxyl terminus of gB was not important for either virion infectivity or egress. To investigate the role of gB in tumorigenicity, we injected nude mice subcutaneously with BCBL-1 cells transfected with anti-gB siRNA, codon-optimized gB and p3xFlag in the presence of Matrigel. BCBL-1 cells transfected with anti-gB siRNA in nude mice produced significantly smaller tumors than the mice transfected with codon-optimized gB and p3xFlag vector. These results provide new insights into the roles of KSHV gB in virion egress, infectivity and tumorigenicity.

STATEMENT OF RESEARCH OBJECTIVES

The overall goal of this research is to gain a better understanding of the role that major viral glycoproteins play in the KSHV lifecycle and pathogenesis. The specific objectives are:

1.) Evaluate the role of gB carboxyl terminus domains in infectivity and viral egress from infected cells by generating several site carboxyl terminal truncations of gB.

2.) Investigate the role of gB in tumorigenesis.

LITERATURE REVIEW

Historical Perspective of Kaposi's Sarcoma-Associated Herpesvirus

Kaposi's sarcoma was first described by Moritz Kaposi in 1872. It has been known as a rare disorder of older men usually of Eastern European, Mediterranean, and/or Jewish origin. Subsequently, three additional forms of Kaposi's sarcoma have also been classified: African-endemic, iatrogenic (in organ and tissue transplant recipients receiving immunosuppressive therapy) and AIDS-KS or (Aluigi et al., 1996; Ambroziak, 1995) Foreman et al., 1997a; (Gao et al., 1996a; 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). It is now established that KSHV infection of the host is common among all four epidemiological forms despite their numerous clinical differences.

In 1994, an innovative technique of representational difference analysis (RDA) was used to identify unique herpesvirus-like DNA sequences. This study led to the discovery of a new human herpesvirus (named Kaposi's Sarcoma-associated Herpesvirus or human herpesvirus 8) from patients infected with both AIDS and KS but not from patients with AIDS who are without KS (Chang et al., 1994). In 1995, primary effusion lymphoma was first described in AIDS patients. Since then, Kaposi's sarcoma-associated herpesvirus (KSHV) has also been associated with multicentric Castleman's disease (MCD), primary pulmonary hypertension and solid/extracavitary lymphomas with similar morphological and immunophenotypical characteristics to those of PEL. The PEL cells are able to support continuous KSHV infection and conditional productive virus replication leading to the study of KSHV.

Herpesviruses are double-stranded DNA viruses and highly disseminated in nature. KSHV and Epstein Barr virus (EBV) are human gamma-herpesviruses known for their

involvement in cancers and cellular proliferation. KSHV can infect lymphocytes, endothelial cells, keratinocytes, human foreskin fibroblast, Vero, 293, HeLa cells, etc. KSHV has aroused substantial scientific interest as a new human DNA tumor virus. Sequence analysis shows that KSHV genome encodes many cellular homologous genes involved in cell cycle regulation, cell proliferation, apoptosis, and immune modulation.

Clinical Significance of Kaposi's Sarcoma-Associated Viruses

Kaposi's Sarcoma (KS)

Kaposi's sarcoma (KS) is the most common AIDS-defining malignancy characterized by multifocal tumor lesions, neo-angiogenesis, proliferation of spindle cells, and infiltration with inflammatory cells and erythrocytes. KS tumors are most commonly found in the dermis but also occur in viscera including liver, lungs and intestines (Hengge et al., 2002). Visceral dissemination results in organ failure and eventual death. The majority of the cells are latently infected with a small subpopulation of lytically infected cells. Both latent and lytic infections make important contributions to KS pathogenesis.

Overall, KSHV infects approximately 40% of gay men and over 60% of Sub-Saharan Africans in many countries. KSHV is necessary for all clinical forms of Kaposi's sarcoma, but it is not sufficient to cause KS. It is now believed that AIDS is very important in KS pathogenesis by causing immunosuppression. Therefore, KS is now the dominant malignancy in many African countries with a high prevalence of AIDS (Dedicoat and Newton, 2003).

KSHV seroconversion could be used to predict the development of the disease (Cesarman et al., 1996b; Gao et al., 1996a; Kedes et al., 1996; Martin et al., 1998; Renwick et al., 1998). These investigations reveal a substantial role for cofactors in KS pathogenesis (Dourmishev et al., 2003). Recent studies suggest that cigarette smoking decreases KSHV risk while drinking is

associated with a higher risk of AIDS-associated KS (Hoover et al., 1993; Mbulaiteye et al., 2006; Nawar et al., 2005).

Classical KS primarily occurs in elderly men of Eastern European, Mediterranean, or Jewish descent (Ablashi et al., 2002; Dourmishev et al., 2003; Moore and Chang, 2003). In the late 1800s, Moritz Kaposi first described the clinical condition of “classical” KS as multifocal pigmented sarcomas (Kaposi, 1872). Classical KS tumors confined themselves to the lower extremities for ten or more years with a benign course (Hengge et al., 2002). The first signs of KS are often the bluish-red macules or papules on the distal lower extremities resulting in multifocal patches and nodules. Those initial patients died of KS, and the clinical presentation of the disease grossly resembles what we currently find in KS patients (Knipe and Philadelphia, 2001). Unlike AIDS-associated KS, HIV-1 co-infection is rare but has been strongly suggested as a cofactor (Vitale et al., 2001). Extensive epidemiological studies demonstrated a high prevalence of KSHV in the Mediterranean contrasted with non-Mediterranean countries. Both sexual and nonsexual modes are probably the two transmission routes of KSHV in classic KS. Saliva has also been suggested as a predominant route in classic KS (Cattani et al., 1999).

AIDS-associated or Epidemic AIDS KS is the most common AIDS-associated neoplasm in homosexual and bisexual men (Feigal, Cheson, and Nelson, 1997). The epidemic AIDS KS, much more so than in the other epidemiologic forms, is an extremely aggressive mucosal progression in the AIDS-infected homosexual and bisexual population (Schwartz, 1996). The prevalence of epidemic KS is approximately 20-fold higher in homosexual AIDS patients than in other HIV infected patients (Goedert, 2000). However, the incidence of AIDS-related KS has been decreased due to the highly active antiretroviral therapy (HAART) available in western

countries (Cattelan et al., 2001). The HIV-1 specific protease inhibitors (PIs) are anti-angiogenic and can inhibit the development of angiogenic lesions.

This disease is most commonly found multifocally and frequently on the upper body, head and neck (Hengge et al., 2002). It quickly evolves to tumors from local lesions and in visceral dissemination resulting in organ dysfunction and high mortality rates. Similar to classic KS, the relative seroprevalence of KSHV is consistent with the KS incidence in HIV-infected populations. It is now well established that a direct correlation exists between HIV-1-infected AIDS pathogenesis and KS progression (Borkovic, 1981; Gottlieb, 1981; Hymes, 1981). The co-infection of KSHV and AIDS contributes to KSHV pathogenesis at multiple levels, including immunosuppression, enhancement of KSHV infection and viral DNA replication, as well as direct effects on KSHV gene expression (Dourmishev et al., 2003).

Recent studies have suggested that sexual transmission of KSHV is the most significant risk factor for HIV-infected populations (Martin et al., 1998). Evidence from a cohort study of seropositivity and seroconversion demonstrated that orogenital rather than anogenital sex is a stronger risk factor for KSHV infection (Dukers et al., 2000). The primary mode of KSHV transmission has not be completely understood, but recent studies have postulated that KSHV transmission via the oral route may also occur among healthy immunocompetent individuals (Cook et al., 2002a; Cook et al., 2002b; Duus and Grose, 2007; 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 posed the highest behavioral risk factor (Osmond et al., 2002). KSHV has also been detected in the saliva of seropositive individuals (Blackbourn et al., 2000; Koelle et al., 1997; Pauk et al., 2000; Vieira et al., 1997), but it is extremely difficult to detect KSHV in semen, with very low reproducibility.

In addition, many other routes of transmission have been studied for KSHV and HIV pathogenesis. A very recent study has inplicated that salivary and possible nosocomial HHV-8 transmission may exist in rural Egypt (Mbulaiteye et al., 2008).

Endemic (African) KS has been found for many decades in Africa, particularly the equatorial region, prior to the worldwide spread of HIV (Oettle, 1962). The highest prevalence of African KS patients have been observed in Zaire, Uganda, and Tanzania (Ablashi et al., 2002). Before the emergence of HIV, African KS primarily affected children of approximately three years of age and young men around age thirty-five (Wabinga, 1993).

Microparticles of silica dust in volcanic soils have been suggested as environmental contributors in children infected with African KS (Ziegler, Simonart, and Snoeck, 2001). Mircroparticles penetrate the skin of the barefoot and then migrate into the lymphatics, leading to inflammation and lymphedema. Aluminosilicates from animal models associated with the direct intralymphatic injection of fine silica particles cause cytotoxic effects on macrophages, leading to subsequent fibrosis within lympho-vessels (Fyfe et al., 1985). Thus, children who walk barefoot could risk the penetration of silica microparticles into their feet, likely resulting in localized immune suppression. This localized immune suppression would provide the proper environment for the development of African KS in KSHV-infected individuals (Ablashi et al., 2002). However, clinical epidemiologic studies of KS in Africa suggest that African KS is most likely dependent on the spread of AIDS, which in turn facilitates the prevalence of endemic KS.

Clinically, KS in Africa is more frequently found in children and women than anywhere else worldwide (Kasolo, Mpabalwani, and Gompels, 1997; Wawer et al., 2001; Ziegler and Katongole-Mbidde, 1996). African KS is typically more aggressive than classic KS but similar to the progression of AIDS-related KS. The endemic KS in Africa with high prevalence in

childhood indicates that KSHV transmission patterns are primarily nonsexual and horizontal, with a few vertical instances. Other evidence also points out that population-delimited routes occur in Africa (Enbom, Linde, and Evengard, 2000; Enbom et al., 2000).

Iatrogenic (transplant) KS with a prevalence of 0.5-5% depending on the patient's country of origin and the type of organ received is becoming a growing concern for solid-organ transplant patients, (Tan and Goh, 2006). It is now well established that KSHV is epidemiologically associated with KS in post-transplant patients, predominantly after renal transplantation (Luppi, Barozzi, and Torelli, 2003). Unlike lymphomas or different epithelial malignancies, iatrogenic KS represents an earlier manifestation of post-transplantation malignancy in recipients (Penn et al., 1993). KSHV infection in transplant KS patients progresses either chronically or rapidly. In general, transplant patients with KS present the manifestation of visceral dissemination of KS with occasional skin lesions (Singh, 2000).

The incidence of iatrogenic KS is 500 times greater than in the general population and its occurrence is introduced by immunosuppressive therapy. However, the remission of KS has been observed in all variants of KS when immunosuppression is removed. More than fifty percent of transplant associated KS patients have had tissue rejection when immunosuppression is reduced or withdrawn (Singh et al., 2000). Extreme ethnogeographic associations have been shown in iatrogenic KS (Penn, 1993). The route of transmission among iatrogenic KS patients has not been fully understood. However, three separate routes have been suggested: donor organ, blood transfusions, and caregivers (Jenkins, Hoffman, and Liegey-Dougall, 2002). Two possible mechanisms of post-transplant KS have also been suggested: KSHV reactivation in patients who were infected before the graft, and KSHV primary infection through infected organ-donor tissues. It has been reported that KSHV reactivation of latent viral infection plays a

greater role in iatrogenic KS in areas of endemic KS, in contrast to nonendemic KS areas, where it is believed that KSHV primary infection is the major cause of post-transplantation KS (Rabkin, Shepherd, and Wade, 1999). Many studies have suggested that KSHV primary infection 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, Klepfish, and Schattner, 2002). To date, there is no precise estimate of KSHV seroprevalence in organ donors, and recipients in different countries. Thus, it is still debated whether or not to screen donors/recipients for KSHV due to the potential risk for KSHV-related disease development.

Pleural Effusion Lymphoma (PEL)

Pleural effusion lymphomas (PELs) or body cavity-based lymphomas (BCBL) were first described in AIDS patients (Cesarman et al., 1995). Compared to KS, PELs manifest the more common neoplasm characterized by irregular cellular expansion of transformed cells (Knipe et al., 2001). PELs are very rare; they are predominately but not exclusively found in HIV infected patients with extremely poor prognosis (Kaplan et al., 1997; Levine et al., 1991). PEL exhibits malignant effusions in the peritoneal, pericardial, pleural, or abdominal cavity, typically without an identifiable tumor mass (Aoki, Jones, and Tosato, 2000; Leao et al., 2002). In rare cases, the solid tumor masses of PELs were also found outside the body cavities (Aoki and Tosato, 2003).

PELs are monoclonal non-Hodgkin's B cell lymphomas derived from late-stage differentiated pre- and post-germinal center B cells. Therefore, any stage of B cell maturation can be found in PELs (Carbone et al., 1998; Matolcsy, 1999). In addition, another study has shown that KSHV was also detected in circulating B cells (Blackbourn and Levy, 1997). PEL tumor cells lack B cell specific surface markers and are commonly dually infected with Epstein-

Barr virus (EBV), which also transforms cells and lead to lymphopreliferative disorders in infected patients (Ablashi et al., 2002).

PEL cell lines derived from PEL have been demonstrated to be the best source for infectious virus production (Moore and Chang, 2003). Unlike KS tumor explants, PEL cell lines stably maintain viral episomes at high copy numbers (50–150 copies per cell). Typically, the majority of PEL cells are latently infected by KSHV; a small percentage (<3%) of cell populations spontaneously undergo lytic reactivation (Renne et al., 1996b). Lytically infected cells can be induced by 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).

Multicentric Castleman's Disease (MCD)

Multicentric Castleman's disease (MCD), also known as multicentric angiofollicular lymphoid hyperplasia, is a rare lymphoproliferative disorder. MCD exhibits a systemic proliferation, including lymphadenopathy and splenomegaly, in part due to raised serum concentration of interleukin 6 (Mikala et al., 1999). Clinically, MCD is classified into two forms: single mediastinal angiofollicular lymph-node hyperplasia localized Castleman's disease and multifocal or multicentric Castleman's disease with multisystem involvement accompanied by generalized lymphadenopathy. Similar to KS, MCD is also characterized by vascular proliferation in the germinal centers (Ablashi et al., 2002). Morphologically, the cells containing KSHV genomes in multicentric Castleman's disease are similar to immunoblasts, with prominent central or marginal nuclei (Judde et al., 2000; Parravicini et al., 2000).

MCD has been reported in close association with KSHV in both HIV-seropositive and HIV-seronegative patients. The precise incidence of MCD is unknown; although, MCD is more common in HIV-positive than HIV-negative individuals. KSHV has been found mostly (greater

than 90%) in HIV-related MCD in AIDS patients (Aoki and Tosato, 2003; Leao et al., 2002). This feature of MCD strongly suggests an important role of KSHV in the pathogenesis of MCD, particularly in AIDS patients (Grandadam et al., 1997). Furthermore, in AIDS related MCD, an increased KSHV viral load is correlated with the exacerbation of MCD symptoms even though no manifest MCD foci were found 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, unlike KS and PEL, not all Castleman's disease has been found to contain KSHV genomes indicating more than one etiologic factor contributing to the disease (Moore and Chang, 2003). In patients co-infected with HIV and KSHV, MCD presents itself in a significantly more aggressive form. The prognosis of MCD in HIV-positive patients generally remains poor with a median survival of 48 months from diagnosis and a 15-fold increased risk of non-Hodgkin's lymphoma (Oksenhendler et al., 2000; Parravicini et al., 1997a; Zietz et al., 1999).

MCD is a polyclonal tumor. In MCD, the majority of the cell mass is composed of uninfected lymphocytes which are recruited to the site of infection by cytokines released from KSHV infected B cells (Dupin et al., 1999; Katano et al., 2000; Parravicini et al., 1997a). In contrast to PELs in which the majority cells are dually infected with KSHV and EBV, MCD usually is negative for EBV (Aoki, Jones, and Tosato, 2000; Little et al., 2001). There is higher number of lytic KSHV genomes in MCD than in PEL implying different KSHV pathogenetic mechanisms between MCD PEL. The different mechanisms of MCD and PEL may be due to different 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).

KSHV Genome Structure and Organization

Many studies of KSHV viral structure have shown that KSHV shares similar structure features with other members of the Herpesviridae family. Typically, the observed size of KSHV virion particles is approximately 100-150nm (Renne et al., 1996b). Like other herpesviruses, KSHV is comprised of four structural elements: 1.) a large linear double-stranded DNA in a cylindrical core; 2.) an icosahedral capsid composed of 162 hexagonal capsomeres; 3.) an amorphous tegument surrounding the capsid, and 4.) a lipid bilayer envelope derived from the host cell (Knipe et al., 2001).

The KSHV genome was determined by sequencing of viral DNA isolated from both a PEL cell line and biopsy specimens of KS. Virion DNA released from BCBL-1 cells treated with phorbol ester (12-O-tetradacanoyl-13-acetate; TPA) revealed that the KSHV genome is comprised of approximately 165kb to 170kb (Renne et al., 1996a; Zhong et al., 1996). Compared to the prototypical herpesvirus HSV-1 genome which is 135 kb, the KSHV genome size is a bit larger. However, the KSHV genome is substantially smaller in size than the viral genome of the human cytomegalovirus (HCMV). Although numerous published results have thoroughly investigated molecular aspects of the KSHV lifecycle further, virion structure and morphology is still unclear due to the difficulty ofcultivating KSHV in either tissue culture or animal systems. Negative stained images of thin sections revealed that KSHV and HCMV capsids are similar. The KSHV nucleocapsid was observed to be approximately 120-150nm, while HCMV nucleocapsid was shown to be 150-200nm (Sarid et al., 1997). It has been shown that the capsid size and structure are consistent with other members of the herpesvirus family by using electron cryomicroscopy (Trus et al., 2001; Wu et al., 2000).

The Core and Organization of the Viral Genome

Like all other herpesviruses, the innermost core of the KSHV particle consists of wound viral DNA genome (Knipe et al., 2001). Herpesviruses possess a linear double-stranded DNA genome with a size range from 120kb to 250kb. DNA purified from intact virions showed that the KSHV genome is approximately 165kb to 170kb by using pulse-field gel electrophoresis (Renne et al., 1996a). Nucleotide sequence data suggests that KSHV is a member of the gammaherpesvirus subfamily, genus Rhadinovirus (gamma-2 herpesvirus), which also includes the new world monkey virus, herpesvirus saimiri (saimiriine herpesvirus 2, HVS). KSHV is the first member of this genus known to infect humans (Moore et al., 1996b).

The linear double stranded genome consists of a continuous 140.5kb central region composed 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 the 20-35kb multirepetitive terminal region containing high GC DNA, which has 84.5% G+C content terminal repeat units (TR) (Neipel, Albrecht, and Fleckenstein, 1997).

Approximately 90 genes have been identified in the KSHV's long unique region, which resembles the genetic organization of herpesvirus saimiri (HVS) (Moore et al., 1996; Neipel, Albrecht, and Fleckenstein, 1997). About 70 conserved genes arranged co-linearly are found in both KSHV and HVS with small intermittent regions contain unique genes of each virus. Corresponding to the previous nomenclature of genes for HVS, all conserved genes have been designated the prefix "ORF" and numbered consecutively from left to right across the viral genome, while the KSHV unique genes within the intermittent regions have been denoted K1 to K15 (Russo et al., 1996). The genetic organization was shown to be highly conserved among

other nonhuman members of the genus rhadinovirus, such as the rhesus rhadinovirus and murine gammaherpesvirus 68. The conserved genes among the rhadinovirus genus function metabolically or catalytically in both virion structure and viral DNA replication, including glycoprotein B (ORF8), DNA polymerase (ORF9), thymidine kinase (ORF21), glycoprotein H (ORF22), DNA helicase-primase complex (ORF40, ORF41 and ORF44), splicing factor (ORF57), polymerase processivity factor (ORF59), p33 (ORF69), thymidylate synthase (ORF70) , serine/threonine protein kinase (ORF36) (McGeoch and Davison, 1999; Moore et al., 1996b; Neipel, Albrecht, and Fleckenstein, 1997; Park et al., 2007; Russo et al., 1996; Santarelli et al., 2008).

KSHV shares both structural and biological features with the Epstein-Barr virus (EBV), the only other human gammaherpesvirus causing human tumors. The two viruses are oncogenic tumor viruses, although KSHV contains none of the EBV-like unique latency genes in cell immortalization and transformation (Knipe et al., 2001; Russo et al., 1996). A clear genetic correspondence was found between KSHV and EBV even though no homologies of latency genes were shown between KSHV and EBV (Moore and Chang, 2001). Both EBV and KSHV utilize B lymphocytes as reservoirs during latency and establish persistent infection in a similar B cell environment. However, a certain set of cellular genes was induced by EBV proteins, and this set of human cDNAs was modified by KSHV that are captured and incorporated themselves into cellular genome (Knipe et al., 2001; Neipel, Albrecht, and Fleckenstein, 1997). Possible functional cross-complementation between members of the gammaherpesvirus subfamily has been suggested by the study of KSHV p33 that shares many similarities with its EBV BELF2 (Santarelli et al., 2008).

The Capsid

Similar to other herpesviruses, the KSHV virion has a multilayered architecture, with a double-stranded DNA genome enclosed within an inner icosahedral capsid, a thick middle tegument compartment containing proteineases, and a lipid bilayer envelope embedded with glycoproteins (Steven, 1997). The capsids are first formed after the initial KSHV replication in the nucleus and mature. The capsids contain linear KSHV genome. Only newly synthesized virions are packaged into the capsids (Knipe et al., 2001; Renne et al., 1996b). Similar to encapsidated HSV-1 DNA, encapsidated KSHV DNA is present within the capsid as a single linear copy encoding the entire viral genome (Booy et al., 1991). Unlike EBV, the KSHV genome is a large closed circular episome during latency, and the viral genome linearizes during virion packaging and replication (Decker et al., 1996). The mechanism by which this packaging occurs has not been fully understood although the linear KSHV genome possibly enters into the capsid through a unique portal vertex (Cardone et al., 2007; Chang et al., 2007; Deng et al., 2007; Deng et al., 2008; Newcomb et al., 2001; Trus et al., 2004). Genetic and biochemical studies have suggested a bacteriophage-like, DNA-packaging/ejection portal complex, located at only 1 of the 12 vertices of the capsid of herpesviruses, which has been demonstrated recently by cryo-electron tomography (cryoET) studies of native KSHV capsids (Nealon et al., 2001). However, the exact arrangement and orientation of the portal complex remains unclear.

The nuclei of infected cells provide a source for biochemical analyses of HSV-1 and HCMV capsid structures (Homa and Brown, 1997); however, it is well recognized that isolating enough amounts of stable capsids in cell culture from both EBV and KSHV for structural studies is extremely difficult. Lytic replication induced by chemical treatments in infected cells offers a stable source of KSHV capsids (Dolyniuk, Pritchett, and Kieff, 1976; Nealon et al., 2001; Wu et

al., 2000). The establishment of a stable source of KSHV capsids has allowed further determination of the proteins and the molecular ratios of the proteins involved in the composition and structure of the capsid (Nealon et al., 2001; Trus et al., 2001; Wu et al., 2000).

Since the 1970s, much biochemical evidence, visualized via electron microscopy and separated by density gradient sedimentation, have demonstrated that herpesviruses form in distinctive capsids during assembly in the host nucleus. The three major capsids were named A capsids, B capsids and C capsids according to the order of their positions on a density gradient. Later protein analysis and negative stain electron microscopy revealed that the capsid shell is a nearly identical icosahedral shell among three classes, and the classes are only differentiated by their inner contents. A capsids are complete void of DNA or any detectable internal structure with a total mass of 200MDa. B capsids contain an inner array of scaffolding protein from ORF17.5 (Nealon et al., 2001) with a total mass of 230 MDa. C capsids are filled with the packed KSHV double-stranded DNA genome only but no scaffolding protein, which was present in the B capsids with a total mass of 300 MDa. Biochemical and mass spectrometry studies showed that the A, B, and C capsid shells are composed of four viral proteins, including the ORF25/MCP (major capsid protein), ORF65/SCIP (small capsomer interacting protein), ORF62/TRI-1 (triplex monomer protein) and ORF26/TRI-2 (triplex dimer protein) (O'Connor and Kedes, 2006). The smallest capsid proteins (SCPs) encoded by ORF65 gene have been found to localize at the outermost regions of the capsid. The localization of SCP suggests that SCP may be important in mediating capsid interactions with the tegument and cytoskeletal proteins during infection (Lo et al., 2003).

Herpesvirus capsids consist of 150 hexameric and 12 pentameric capsomers formed exclusively from the KSHV-ORF25 product/MCP (the major capsid protein). The majority of

the capsid's mass can 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 heterotriplexes 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 deenveloping intact virions; furthermore, type C capsids are (about 10-15% of total capsids produced) produced the least from induced BCBL-1 cells (Gibson and Roizman, 1972; Schrag et al., 1989). The small percentage of type C capsids produced from BCBL-1 cells may provide an explanation for the low infectivity of KSHV in experiments using this cell line as a source of virus stock (Nealon et al., 2001). Cryo-electron Tomography (cryoET) and three-dimensional reconstruction techniques have been powerful tools in studying the structure of KSHV capsids. KSHV capsids directly visualized by cryoET reveal a heterogeneous collection of similar but non-identical particles containing different amounts and configurations of SCAF, while neither A nor C-capsids showed structural variation (Deng et al., 2008). The highly variable SCAF may represent the existence of different intermediates during the KSHV capsid assembly and maturation.

The Tegument

The tegument of herpesviruses is a proteinaceous region contained between the outer surface of the capsid and the underface of the virion envelope. The tegument is various in both size and composition (Roizman, 1974). The tegument can be distributed asymmetrically and symmetrically. There is less tegument that are more symmetrically arranged in perinuclear virions than in virions in cytoplasmic vesicles that contain tegument distributed more asymmetrically (Falke, Siegert, and Vogell, 1959).

The studies of the structure and function of the herpesvirus tegument, particularly about the KSHV tegument, have provided limited information. Tegument proteins play important roles in various aspects of the virus life cycle, the early events of infection and virion egress. Certain herpesvirus tegument proteins have been shown to regulate the viral lifecycle, penetration of the virus into cells and viral replication (Knipe et al., 2001).

KSHV ORF45 has been found in the tegument area of virions and plays a critical role in the KSHV lytic replication cycle (Zhu and Yuan, 2003). It is an immediate-early protein, highly phosphorylated. OFR45 is expressed immediately after the virus enters the lytic cycle. Its expression level increases as lytic viruses replicate and remain at a high amount through the late stage (Zhu et al., 2005; Zhu, Cusano, and Yuan, 1999; Zhu and Yuan, 2003). Previously, ORF45 protein has been shown to interact with cellular IRF-7 (interferon regulatory factor 7) and suppress its phosphorylation and nuclear accumulation, indicating a role of ORF45 in inhibiting the host anti-viral response (Zhu et al., 2002). IRF-7 regulates the type I Interferons (IFNs) and induction of IFN-α and IFN-β (Samuel, 2001; Stark et al., 1998). ORF45 has also been reported to interact with different cellular pathways that are essential for KSHV lytic replication (Zhu et al., 2006). In addition to tegument proteins, mRNAs, spanning more than one kinetic class, have been shown to encapsidate into the virus particles of alpha and beta herpesviruses (Bresnahan and Shenk, 2000; Sciortino et al., 2001; Sciortino et al., 2002). 11 virally encoded RNAs in KSHV virions have been identified and are critical for the establishment and maintenance of KSHV latency (Bechtel, Grundhoff, and Ganem, 2005; Cai et. al., 2005). The mechanism by which KSHV also possesses mRNAs within intact virions is not fully clear.

The Envelope

The outermost layer KSHV virion envelope is made up of altered infected cellular membrane. The KSHV envelope is embedded with several glycoproteins designated as gB, gH, gpK8.1, gL, gM, gN and others (Koyano et al., 2003). A number of herpesviral glycoproteins play important roles throughout the viral lifecycle, including attachment, penetration, egress, and virus-induced cell fusion and tumorigenicity (Knipe et al., 2001).

Kaposi's Sarcoma-Associated Herpesvirus Lifecycle

Viral Attachment and Entry

Attachment of many enveloped viruses is a multistep process mediated by interactions between specific viral glycoproteins with receptors on the surface of the target cell. Entry is the most critical step in the KSHV life cycle and greatly determines the pathogenesis of members of the herpesvirus family. Entry of herpesvirus into a susceptible cell involves two steps: the first step is virus binding to cell surfaces via viral envelope glycoproteins and cellular receptors (Spear and Longnecker, 2003). The second step is fusion of the viral envelope with cellular membranes, releasing viral capsids into the cellular cytoplasm. Alternatively, virions are internalized into endosomes with low pH in which the mildly acidic environment triggers viral glycoprotein-induced fusion with the endosomes.

Binding Receptors

The initial binding to target cells of KSHV is mediated via KSHV envelope-associated glycoprotein gB and gpK8.1A, 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). In addition, KSHV interacts with the alphaVbeta3 integrin via the Arg-Gly-Asp (RGD) motif of its glycoprotein B (Garrigues et al., 2008). Heparan sulfate proteoglycans

covalently attach to glycosaminoglycans, abundantly distributed on the surface of mammalian cell membranes (Stringer and Gallagher, 1997). Proteoglycans have key roles in various fundamental cellular processes including cell-to-cell adhesion, cell-to-matrix adhesion, cellular motility, cellular growth and cellular signaling transduction (Kjellen and Lindahl, 1991; Rostand and Esko, 1997). The presence of heparan sulfate is not essential for virus entry, but it greatly increases the efficiency of virus entry into cells (Banfield et al., 1995).

Herpesvirus viral attachment to cell surfaces is inhibited by soluble heparin, a molecule having a structure similar to heparin sulfate (Akula et al., 2001b). Furthermore, the inhibition of KSHV particle entry into the cell cytoplasm by heparan is in a dose-dependent manner (Akula et al., 2001a; Birkmann et al., 2001; Wang et al., 2001a). For the first time, it was shown that KSHV infected B cells are capable of preferentially using cellular (αV) or viral (gB) receptors to specifically bind cells, depending upon the stage of the cell cycle and infection. Using the Matrigel system, KSHV infected PEL cells can attach to extracellular matrix proteins. This attachment occurs preferentially in cells from S phase or cells from S phase actively supporting a lytic infection, respectively (Dyson et al., 2008).

Entry Receptors

The integrin receptor family has been indicated by a number of other viruses to be an entry pathway for infection. Integrins control a wide variety of cellular functions that include cell survival, gene expression, cell cycle, actin dynamics, cell migration, angiogenesis, including cell attachment (Martin et al., 2002).

Several proteins have been reported to function as HHV-8 entry receptors. KSHV infects a variety of cell types from different tissues and species, including B, T, endothelial, epithelial, fibroblast, keratinocyte cells, owl monkey kidney and baby hamster fibroblast cells

(Cerimele et al., 2001; Ciufo et al., 2001; Flore et al., 1998; Foreman et al., 1997; Gao, Deng, and Zhou, 2003; Kliche et al., 1998; Mesri et al., 1996). This broad tropism for different cell types indicates a specific KSHV fusion-entry receptor(s) on the target cell surface. The cystine transporter, xCT, was identified as a KSHV fusion-entry receptor by functional cDNA selection (Kaleeba and Berger, 2006).

Previous studies have shown that KSHV can successfully infect human monocytes and macrophages both in vitro and in vivo. It has been recently reported that KSHV uses DC-SIGN (Dendritic cell-specific ICAM-3 grabbing nonintegrin; CD209) to enter macrophages and dendritic cells derived from monocytes (Rappocciolo et al., 2006). DC-SIGN is a required receptor for HHV-8 infection of myeloid DCs and macrophages. Furthermore, infection of B cells was reduced by blocking DC-SIGN leading to the conclusion that the expression of DC-SIGN is crucial for productive KSHV infection and replication in B cells (Rappocciolo et al., 2008). However, the KSHV viral proteins that interact with the xCT and DC-SIGN receptors have not yet been identified. Moreover, a very recent study has shown that KSHV, as a human DNA tumor virus, specifically promoted TLR3 in human monocytes which until now has only been shown to be associated with the recognition of RNA viruses. In this study, siRNAs directed against TLR3 significantly inhibited the ability of KSHV to up-regulate IFN-β1 and CXCL10 (West and Damania, 2008).

The integrin αVβ3 is a cellular receptor mediating both the cell adhesion and entry of KSHV into target cells through binding the RGD motif of virion-associated gB. The αVβ3 is widely expressed in proliferating endothelial cells, arterial smooth muscle cells, and certain populations of leukocytes and tumor cells. The integrin αVβ3 mediates the adhesion of cells to a number of extracellular matrix proteins containing RGD motifs, including vitronectin and

fibronectin, and being responsible for mediating cell-cell interactions through binding to cell-associated glycoproteins containing RGD motifs. In addition, αVβ3 has been reported as a receptor for the entry of target cells during the infection process by other viruses (Plow et al., 2000). In KS tumors, αVβ3 is highly expressed on KS tumor spindle cells and in neovascular endothelial cells and B cells that infiltrate KS tumors (Kaaya, Mwangi, and Ouna, 1996; Salcedo and Patarroyo, 1995; Uccini et al., 1994). These cells are also consistently infected with KSHV in KS lesions suggesting a central role for αVβ3 in KSHV infection and in the pathogenesis of KS.

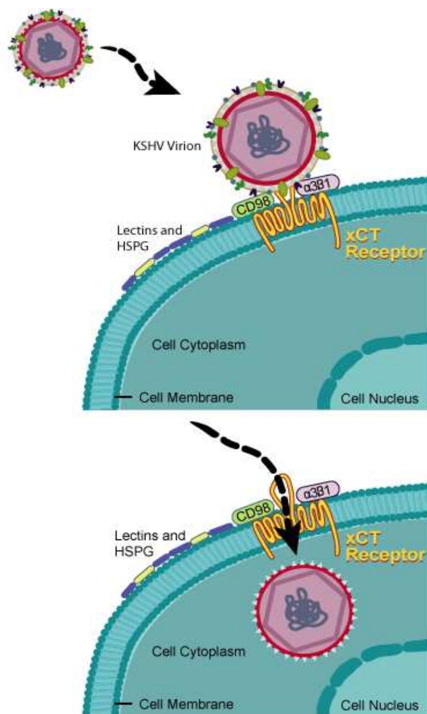

Figure 1.1: KSHV Fusion and Entry. This illustration shows how KSHV fuses to and enters a human cell after binding to the protein xCT (Berger EA. *Science*. 2006 Mar 31; 311(5769):1921-4.)

KSHV Latency

One of the striking features of KSHV is the lifelong latent infection in host cells that modulates the host immune response. Similar to other herpesviruses, KSHV has two modes of infection: latent (nonproductive) infection in which the viral genome persists in its host cell but with dramatically restricted gene expression and without cell destruction, and lytic (productive)

infection with progeny virions and the destruction of the host cell (Zhong et al., 1996). In vivo, only less than 3% of infected cells in KS lesions or KSHV-positive lymphomas display evidence of lytic KSHV gene expression (Renne et al., 1996b). Most commonly, KSHV infection remains in the latent state with very few genes expressed. Host cell conditions play an important role in regulating both lytic and latent infections of target cells (Bryan, Dyson, and Akula, 2006; Mercader et al., 2000). KSHV latent and lytic DNA replications are 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).

Lytic replication is necessary for the initial infection and replication of the virus in the host. A productive lytic infection of herpesviruses leads to large amounts of virions and cell death via lysis. The lytic cycle has traditionally been thought not to contribute directly to oncogenesis because cells that enter this program invariably die. KSHV readily transforms cells leading to tumorigenesis in vivo (Boshoff, 2002). Lytic replication involves gene expression of virtually the entire viral genome. Viral DNA replicates and produces infectious viral progeny leading to the death of host cells. Lytic replication can be induced from latency by the induction of the transcription factor RTA (replication and transcription activator) (Lukac et al., 1998; Sun et al., 1998). A recent study has suggested that KSHV RTA can regulate viral replication by promoting degradation of the suppressors, including K-RBP and HEY1 (Yada et al., 2006; Yang and Wood, 2007; Yang, Yan, and Wood, 2008). K13 has been indicated to be able to block KSHV lytic replication via decreasing vIL-6 and hIL-6 expression (Zhao et al., 2007). In addition, the induction of lytic replication can be also achieved by the treatment of latently

infected cells with phorbol esters or sodium butyrate or by transfection with consititutively active RTA alleles.

EBV latent infection and the concomitant expression of the latent viral genes are essential for the development of EBV-related lymphoproliferative disorders (Griffin, 2000). Like 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). Latent infection of KSHV is default in most host cells. Viral DNA exists as a circular episome in the nucleus and no progeny virions are produced. In latency, viral gene expression is only limited to seven genes, including: 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). These latent genes have been shown to manifest both growth transforming and cell cycle-deregulating properties (Cotter and Robertson, 2002; Komatsu et al., 2002).

LANA-1 (latency-associated nuclear antigen) encoded by ORF73 is perhaps the most important latent gene expressed during the latent cycle. LANA-1 is highly expressed in all latently infected tumor cells, indicating that LANA-1 plays a critical role in the maintenance of latent KSHV infection (Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997). LANA-1 has been shown to be a multifunctional protein and serves roles as the following: 1.) binds transcriptional corepressors (SAP30, Sin3A, CIR), 2.) possesses a nuclear localization motif, 3.) binds KSHV TR DNA cooperatively and supports terminal repeat (TR) directed viral replication, 4.) associates with host cellular chromatin proteins and stays on the chromosomes during cell division, 5.) binds and inhibits p53 resulting in the inactivation of p53-dependent promoters, 6.) binds retinoblastoma protein RB1 leading to the induction of E2F-dependent genes, 7.)

transcriptionally activates EBV latency promoters, 8.) binds HIV-1 Tat, and 9.) binds to KSHV-Rta by its direct binding to RBP-Jkappa (Dourmishev et al., 2003; Gonzalo et al., 2005; Lan, Kuppers, and Robertson, 2005; 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., 1996a; 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 1162 amino acids and is identified as a 222–234 kDa phosphoprotein with an acidic internal repeat domain flanked by a carboxyl-terminal domain and an amino-terminal domain by Western blot analysis (Fakhari et al., 2006; Kellam et al., 1997; Rainbow et al., 1997). LANA-1 displays a punctuated nuclear distribution pattern in immunohistochemical experiments (Gao et al., 1997). LANA-1 can also activate 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 episomes before each cell division and then successfully segregate into progeny cells.

KSHV latently infected cells harbor several copies of 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. 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); thus showing a necessary and sufficient role for

LANA-1 in KSHV episome maintenance in transfected cells (Ballestas, Chatis, and Kaye, 1999; Ballestas and Kaye, 2001). A short region in the C terminal part of LANA-1 has been demonstrated to be required LANA's abilities, such as binding to and replication of viral episomes, modulation of transcription, and interaction with the members of Brd chromatin binding proteins Brd2/RING3 and Brd4s (Ottinger et al., 2006; Viejo-Borbolla et al., 2005; You et al., 2006). 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 (Cotter, Subramanian, and Robertson, 2001; Krithivas et al., 2002; Mattsson et al., 2002). 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, 2001; Fejer et al., 2003; Grundhoff and Ganem, 2003; Hu, Garber, and Renne, 2002; Lim et al., 2002). LANA-1 can mediate the inactivation of lytic cycle associated genes in the neighborhood of the terminal repeats through local interaction with SUV39H1. SUV39H histone methyltransferases regulate a unique histone H3 lysine9 (H3–K9) methylation. Trimethyl H3K9 is a marker of constitutive heterochromatin. 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). LANA-1 also recruits de novo DNA methyltransferases DNMT3A that in turn bind to another euchromatic histone methyltransferase SETDB1/ESET, which can also trimethylate H3–K9 (Li et al., 2006; Shamay et al., 2006). Unlike other KSHV promoters tested thus far, LANA promoter is not affected by tetradecanoyl phorbol acetate or viral lytic cycle functions. However, it is subjected to control by LANA itself and cellular regulatory factors, such as p53.

Viral Gene Transcription and Expression

Traditionally, transcription of herpesvirus genes are tightly regulated and sequentially ordered in a cascade fashion (Knipe et al., 2001). The sequentially ordered cascade of transcription occurs via a simultaneously and sequentially ordered viral protein cascade which modulates viral gene expression. Herpesviruses use 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 few 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 inconsistent with the establishment of KSHV transformation of the infected cell. A substantially 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., 2001). 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 infections of cultured endothelial cells have also manifested a similar “hodge-podge” pattern of latent and lytic gene expression (Ciufo 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 designations of individual KSHV genes as either latent or lytic genes. Typically, PEL cell lines (in which every cell in the PEL culture is infected with KSHV) carry 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 class I KSHV-ORFs have corresponding sequence homologs in HVS (Nicholas, Cameron, and Honess, 1992; Thome et al., 1997), and their respective expressed proteins 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 class II gene transcription includes mRNAs which are detected in variable abundance of high, moderate, or low in cultures grown under standard growth conditions without phorbol esters. In addition, the 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

contain 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), thus 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 containing regulatory genes that are also referred to as "latency islands" (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 phorbol esters (Sarid et al., 1998). Class III transcripts include lytic genes which are transcribed during active infection and are necessary for efficient viral lytic replication and virion particle production including the following transcripts: ORF 25 (major capsid protein), ORF 6 (DNApolymerase), and ORF 22 (glycoprotein H) (Sarid et al., 1998). Many class III genes 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 virus 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 gamma herpesviruses 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 allows for the virus to evase host immune surveillance and establishes long-term and 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). To establish latent infection in susceptible cells, viral reactivation must overcome a stringent regulation of viral gene expression, since KSHV viral gene expression is highly limited and tightly controlled. KSHV spontaneously reactivates in infected cells and the process of viral lytic replication begins. 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).

Many recent studies have demonstrated that cell cycle progression has a role in reactivation of KSHV infection (Bryan, Dyson, and Akula, 2006; McAllister et al., 2005). A direct correlation between cell cycle progression, KSHV reactivation and cell-surface topology has also been observed (Whitman et al., 2007). In this study, the KSHV-infected cells in G0/1 phase had finger-like projections on their surface, while cells in S phase had a relatively smooth cell surface. Furthermore, a very recent study has suggested that cell cycle has an effect on the expression of cellular receptors utilized by KSHV. This study indicated that KSH-infected human B cells can preferentially use cellular αV integrin or viral receptors to specifically bind cells, depending upon the stage of the cell cycle and infection (Dyson et al., 2008).

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; Muralidhar et al., 2000; Rainbow et al., 1997; Sadler et al., 1999; Saveliev, Zhu, and Yuan, 2002). The expression profile of these latent genes serves a crucial role in the maintenance of latency and cellular transformation. 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). Evidence over the years clearly suggests roles for reactivation and/or lytic replication in the pathogenesis and induction of KS tumors: 1.)

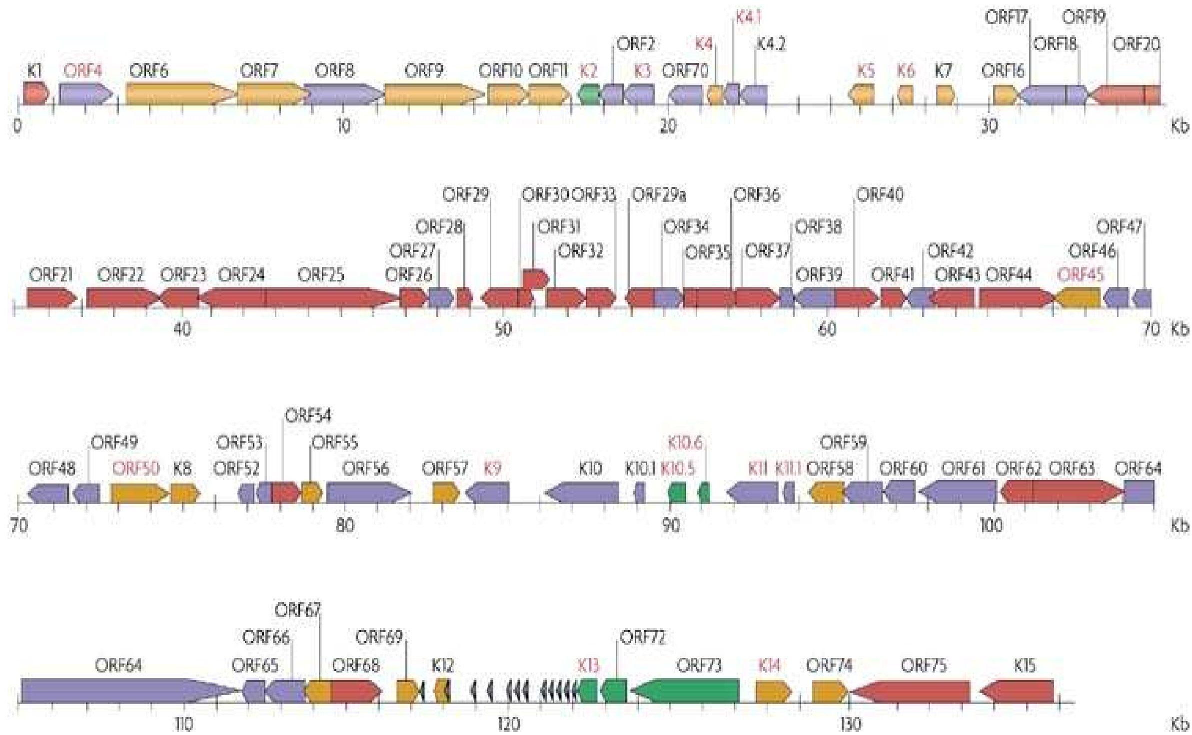

Figure 1.2: KSHV Gene Map. The virus can then either enter a latent program, in which the viral episome is maintained and no virions are produced, or enter a lytic program, in which viral particles are produced and the host cells are lysed. During the latent program, a small number of proteins (indicated in green) are expressed to maintain the viral episome, to avoid host antiviral immune responses and to provide a growth advantage to infected cells. Latently infected cells can be induced to enter the lytic cycle under specific physiological conditions. The factors that regulate the switch from the latent to the lytic cycle have not yet been fully characterized, but they involve the viral proteins replication and transcriptional activator (RTA; encoded by open reading frame 50 (ORF50)) and ORF57, as well as recombination signal binding protein for immunoglobulin J region (RBP-J), a transcription factor downstream of the Notch signalling pathway. Lytic replication leads to extensive viral gene expression. Lytic gene expression is tightly regulated (viral genes transcribed early upon reactivation are indicated in yellow, those transcribed during intermediate kinetics in orange, and those transcribed during late kinetics in red). KSHV-encoded microRNAs are indicated by grey arrowheads (Laurent Coscoy. *Nature Reviews Immunology*. 2007 May, 7,391-401).

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); 4.) both KS tumor cells (typically manifest a latent KSHV infection) and KSHV infected spindle cells (also typically manifest in latent KSHV infection), KS tumor cells and KSHV infected spindle cells consistently undergo spontaneous reactivation of concomitant lytic replication from latent cells in a small population (Reed et al., 1998; Staskus et al., 1999; Staskus et al., 1997; Zhong et al., 1996). Viral lytic reactivation and DNA replication are considered crucial in KSHV spread from the lymphoid reservoir B cells to endothelial cells and the sequential KS spindle cell formation (Offermann, 1999). Hence the reactivation mechanisms regulating the switch from latent to lytic replication in KSHV infected cells serve an essential role in the development of KS and the KSHV-related lymphoproliferative disorders.

Regulation of Transcriptional Activation

Upon chemical induction, KSHV expresses viral transcription activator proteins such as ORF50 and K8, which are important for the induction of lytic replication (Zhu, Cusano, and Yuan, 1999). The viral immediate-early transactivator Rta/Orf50 is necessary and sufficient for virus reactivation and lytic replication (Gradoville et al., 2000; Lukac, Kirshner, and Ganem, 1999; Lukac et al., 1998; Sun et al., 1998). Initially, KSHV-Rta was 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 and expressed earlier than K8 (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 lead 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 (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). The 691 amino acid Rta has been shown to be highly post-translationally modified and extensively phosphorylated (Lukac, Kirshner, and Ganem, 1999; Lukac et al., 1998). A number of transfection experiments have indicated 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 and Alwine, 1999; Lukac, Kirshner, and Ganem, 1999; Lukac et al., 1998; Song et al., 2001) 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. When transfected into BCBL-1 cells, this Rta-specific dominant negative inhibitor of transactivation Rta would effectively suppress spontaneous lytic reactivation from latency along with reduction 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., 2002a; Liang et al., 2002; Lukac et al., 2001; Song et al., 2001; Song et al., 2002). The major targets of Rta transactivation are considered essential for lytic replication. Rta has been shown to transactivate the following genes which are considered essential for viral subversion of the 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., 2001). 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. When demethylized, the promoter latent KSHV genome is induced and lytic replication begins (Chen et al., 2001). Furthermore, more evidences have shown that many cellular and viral proteins, including K-RBP, are capable of repressing RTA-mediated transactivation and KSHV lytic replication. Herpes simplex virus type 1 transactivator ICP0 and human cytomegalovirus transactivator pp71 also stimulate the degradation of repressors in the host. Recently, KSHV RTA has been demonstrated to regulate lytic replication by promoting the degradation of the repressor K-RBP (Yang, Yan, and Wood, 2008). Therefore, silencer degradation by viral transactivators may be a common mechanism for regulating the lytic replication of herpesviruses.

K-bZIP, a leucine zipper-containing transcription factor encoded by ORF K8 can modulate viral and cellular gene expression and affect host cell functions. K-bZIP nuclear protein (Zhao et al., 2007) interacts with and represses RTA-mediated transactivation of viral

promoters, including that of the K8 gene. A recent study has suggested that a new role for K-bZIP is that it competitively binds to the interferon-responsive factor 3 elements leading to immune evasion (Lefort et al., 2007).

Viral DNA Replication

As a gamma-herpesvirus, KSHV possesses both latent and lytic replication cycles (Miller et al., 1997; Renne et al., 1996). However, latency expresses only a small 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 trans-acting 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 facilitates its lytic encoded replication proteins (Miller et al., 1997; Renne et al., 1996a). During the viral lytic replication, 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 aspects from the latent DNA replication. First, 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 known as "head-to-tail" concatemers (Knipe et al., 2001). In contrast to this

rolling circle replication during viral lytic phase, latent DNA replication occurs simultaneously with the host cell division and sequentially maintains a stable and low number of viral episomes. Secondly, lytic herpesviral DNA replication requires virally encoded replication proteins with its own DNA polymerase and related 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 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; for example they are rich in AT regions. These AT-rich regions are presumably sites where DNA is melted or unwound, numerous transcription factor-binding sites, and promoter enhancer elements 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, while 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 the 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). Six core replication proteins are required for ori-Lyt-dependent DNA replication spanning the three herpesviral subfamilies. These proteins include DNA polymerase, processivity factor, helicase, primase, primase-associated factor, and ssDNA binding protein (Knipe et al., 2001). The KSHV genome encodes a set of six genes which have various levels of homology to their respective core replication gene counterparts in EBV, HSV, and HCMV. The six KSHV core replication proteins are listed as follows: 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 is 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). The KSHV RTA has been shown to be sufficient for KSHV viral reactivation but indispensable for viral DNA replication. Rta serves as the major transactivator of gene expression recruiting the pre-replication complexes to ori-Lyt DNA. RTA interacts with the

RRE and bZIP (K8, RAP), which binds to a cluster of C/EBP binding motifs with the aid of C/EBP alpha (Lin et al., 2003; Wang et al., 2006). Several cellular factors associated with ori-Lyt have been identified by using DNA affinity purification and mass spectrometry. These cellular proteins that bind to KSHV ori-Lyt include topoisomerases (Topo) I and II, MSH2/6, RecQL, poly (ADP-ribose) polymerase I (PARP-1), DNA-PK, Ku86/70 autoantigens, and scaffold attachment factor A (SAF-A) (Wang et al., 2008).

KSHV Glycoproteins and Their Putative Functions

The Kaposi's sarcoma-associated herpesvirus genome encodes several glycoproteins, some of which possess significant homology with 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, KSHV encodes glycoprotein gpK8.1A, gpK8.1B, K1, vOX2 (K14) and K15 during lytic replication with no counterparts in other herpesviruses (Chandran et al., 1998a; Chandran et al., 1998b; Chung et al., 2002; Neipel et al., 1997; Schulz, 1998b). Glycoproteins serve various functions at key points along viral replication including 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.

<u>Glycoprotein B (gB)</u>

Glycoprotein B (gB) is one of the most conserved glycoproteins across all subfamilies of herpesviruses. Moreover, the carboxyl-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). gB is essential for members of the alpha- and betaherpesvirus subfamilies. gB expresses on the surface of host cells or on virion envelopes as a homodimeric membrane protein

(Claesson-Welsh and Spear, 1986; Claesson-Welsh and Spear, 1987; Horsburgh et al., 1999; Ligas and Johnson, 1988; Roop, Hutchinson, and Johnson, 1993; Spear and Longnecker, 2003).

gB is the product of the KSHV-ORF8 (Russo et al., 1996). The KSHV-gB molecule is classified as a type I integral membrane protein, 845 amino acids in length, consisting of four domains: 1.) signal peptide domain, 2.) ectodomain (exposed towards the outside of the cell), 3.) transmembrane domain, and 4.) carboxyl-tail domain (located within the cytosol). The first putative signal sequence of 23 amino acids (a.a) of KSHV-gB is an extracellular domain containing multiple glycsylation sites and multiple hydrophobic regions, of which the carboxyl-most terminal region keeps gB in the membrane (Baghian et al., 2000; Pertel, 2002; Pertel, Spear, and Longnecker, 1998). The amino acids 733 through 752 constitute the transmembrane domain, the amino acid 703 is ectodomain and the 93 a.a composes carboxyl terminal tail (Wang et al., 2003). The carboxyl tail of KSHV-gB displays similar characteristics to the cytoplasmic terminal domain of HSV1-gB, in which the hydrophilic amino acid residues in this region are positively charged (Pellett et al., 1985; Pertel, Spear, and Longnecker, 1998). 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). Glycosylation inhibition experiments have clearly shown that KSHV-gB contains predominantly N-linked carbohydrates (Baghian et al., 2000; Pertel, Spear, and Longnecker, 1998). In virions produced from BCBL-1 cells, the virion envelope-associated gB can be detected on the surface of both infected cells and the virion envelope (Akula et al., 2001a; Baghian et al., 2000). Virion envelope-associated gB has also been shown to have strong affinity for heparan by experiments in which KSHV gB binds to heparan-agarose beads with a biotinylated peptide of a gB-heparan

binding domain (gB-HBD peptide consisted of the following 108-117 gB-a.a. sequence: HIFKVRRYRK binds) in a dose-dependent manner to BSA-Heparan coated 96 well plates (Akula et al., 2001a).

A number of studies indicate that gB plays important roles in virion attachment and virus entry into susceptible cells: HSV-1 mutant viruses lacking gB are not able to enter into cells (Cai et al., 1987), this is because of the lack of post-attachment 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 occur, causes 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, deletion of the final 59 a.a of the KSHV-gB carboxyl tail (termed gB-Mut) substantially enhanced cell-to-cell fusion (Baghian et al, personal communication). 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. Interestingly, our work on siRNA-mediated silencing of gB carboxyl terminal truncations showed that the carboxyl terminus of gB is indispensable for virus egress from infected cells and infectivity. KSHV virions incorporate gB in the viral envelope, which is important for attachment to cell surfaces and entry via the RGD motif.

The $\alpha3\beta1$ integrin was first implicated (Akula et al., 2001) as 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). The RGD motif (27-29 a.a.) is immediately behind the KSHV-gB signal peptide. Interestingly, not all herpesvirus gBs possess the gB-integrin interaction since there has been no other gB in the entire Herpesviridae family that has a similar conserved RGD motif. Integrin $\alpha3\beta1$ is broadly expressed and has been detected on all cells susceptible to infection by HHV-8, including human foreskin fibroblasts (HFF) and B, epithelial, endothelial, and 293 cells (Akula et al., 2002; Plow et al., 2000; Wu and Dedhar, 2001). It is also very interesting that at similar concentrations used for the above mentioned virus neutralization experiments, RGD peptides, RGD-gB antibodies, anti-integrin antibodies and soluble integrin $\alpha3\beta1$ proteins could not inhibit KSHV binding to HFF cells while soluble heparan almost completely blocked viral attachment. These results suggested a post-attachment role for the gB/integrin interaction, in which gB binding to the integrin receptor was able to facilitate viral entry into susceptible cells (Akula et al., 2002). Furthermore, the inability of these molecules to completely neutralize viral infectivity implied the potentially other cellular receptors utilized by KSHV for entry. However, a recent study has indicated that cell activity was not correlated with surface expression of integrin $\alpha3\beta1$, suggesting the presence of a putative KSHV fusion-entry receptor (Kaleeba and Berger, 2006). A very recent study has demonstrated that $\alpha V\beta3$ is a cellular receptor mediating both the cell adhesion and entry of KSHV into target cells through binding the RGD motif of virion-associated gB (Garrigues et al., 2008). Envelope-associated KSHV-gB clearly binds to both

heparan sulfate receptors (HS) and integrin αVβ3 which are located on the surface of target cells, thus two different motifs (HBD and RGD) of KSHV-gB mediates KSHV virions binding via HS and viral entry via integrin αVβ3 cellular receptor, respectively (Akula et al., 2001a; Akula et al., 2002; Akula et al., 2001b; Garrigues et al., 2008). Interestingly, the binding of soluble gB proteins to target HFF cells induce morphological changes, e.g., cell rounding and detachment, while 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).

KSHV K8.1

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., 2001a). Consistent 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., 2001a). 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 αVβ3 membrane receptors through a RGD motif, suggesting that integrins function as cellular receptors for KSHV entry (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 a.a protein containing a signal sequence, transmembrane domain and four N-glycosylation sites. The K8.1B cDNA encodes a 167 a.a

glycoprotein sharing similar amino acids and carboxyl termini with K8.1A but contains an in-frame deletion (Chandran et al., 1998a; Raab et al., 1998). K8.1A is the predominant form detected within infected cells and the virion envelopes (Zhu, Puri, and Chandran, 1999). One of the striking features of the K8.1 gene is that it is co-linear to the EBV major glycoprotein gp350/220 (Gong and Kieff, 1990), gp150 of murine gammaherpesvirus 68 (MHV 68) (Stewart et al., 1996), herpesvirus saimiri (HVS) ORF 51 gene and the BOEFD1 gene of bovine herpesvirus-4 (BHV-4) (Albrecht et al., 1992; 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). By using the recombinant KSHV BAC36 system, glycoproteins K8.1 have been indicated to be dispensable for viral entry (Luna et al., 2004).

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; Lomonte et al., 1997; Mukai et al., 1997). 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 of the gH, gL, and gp42 complex formed in EBV is required for the infectivity of B cells; although, gp42 is not required for the infectivity of epithelial cells (Li, Turk, and Hutt-Fletcher, 1995).

Many studies have shown that gH is the only viral fusion glycoprotein displaying typical structural-functional features. Coiled-coil domains in gB and gH have been indicated to be associated with human cytomegalovirus membrane fusion (Lopper and Compton, 2004). It has been reported that the ectodomain of HSV-1 gH contains a hydrophobic membrane alpha-helix (residues 377 to 397) with attributes of an internal fusion peptide (Gianni et al., 2005; Gianni, Menotti, and Campadelli-Fiume, 2005). Moreover, heptad repeat 1 (HR, residues 443 to 471) with a high propensity to form a coiled-coil located downstream of this alpha-helix and plays critical role in virus entry and fusion (Gianni et al., 2006a). HR-2 of gH interacts with HR-1 and serves critical roles in virus entry and fusion. Complex formation between HR-1 and HR-2 is independent of the presence of adjacent gH sequences and of additional glycoproteins involved in entry and fusion (Gianni et al., 2006b). Hydrophobic alpha-helices 1 and 2 of gH are capable of interacting with lipids and promoting virus infection and fusion (Gianni et al., 2006a).

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 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 easily detected during lytic replication induced by TPA in BCBL-1 cells (Naranatt, Akula, and Chandran, 2002).

KSHV-gL is independently expressed on cell surfaces. However, independent expression of gH without gL causes gH mis-folding and aggregation of gH in the ER. In contrast, co-expression of KSHV gH with 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 against either gH or gL do not inhibit KSHV binding, while 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 KSHV-ORF39 and KSHV-ORF53 encode gM and gN, respectively. The gM and gN are conserved throughout the entire family of Herpesviridae. Immunoprecipitation experiments have shown that both KSHV-gM and gN are N-glycosylated and form heterodimers. The heterodimers formation between gM and gN is similar to the heteroduplex formation in other herpesviruses (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 46kDa and 80kDa. The molecular sizes are reduced to 39kDa and 71kDa with the addition of tunicamycin (an inhibitor of N-glycosylation). The glycosylated form of gN is approximately 26kDa and upon tunicamycin treatment the unglycosylated form is approximately 18kDa. gN has shown to be required for proper post-translational modification and transport of gM to the cell surface (Koyano et al., 2003). In addition, gM was present on the surface of the virion envelope as indicated by 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 cell fusion assay of HSV-1 and Mo-MuLV (Molony murine leukemia virus) (Koyano et al., 2003).

Glycoprotein OX2 (vOX2)

Viral genomic analysis of KSHV suggests that ORF K14 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., 2000; Wright et al., 2003). A study has indicated that the immunosuppressive effect of CD200/CD200R interaction may be associated with T-cell; since the receptor CD200R is also present on the surface of certain T-cells (Wright et al., 2003). CD200 possesses a small carboxyl-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 similar to both cellular OX2 (278 a.a) and RRV R14 (253 a.a) (Alexander et al., 2000; Searles et al., 1999).

The predicted KSHV vOX2 contains a typical signal peptide sequence at its amino-terminal region and five potential N-glycosylation sites in its extracellular domain. ORFK 14 (vOX2) exhibits a low 40% DNA sequence identity to cellular OX2. vOX2 binds to the receptor CD200R with almost identical affinity and kinetics as the cellular OX2 (Chung et al., 2002). The c-OX2-OX2R interaction delivers a restrictive signal to macrophages thus limiting macrophage activation. Subsequently, blocking of this interaction with anti-OX2R antibody

exacerbates tissue damage in inflammatory sites (Wright et al., 2000). CD200R on the surface of macrophages can interact with vOX2 expressed on the surface of lytic KSHV-infected cells. It has also shown that the KSHV vOX2 can inhibit the TNF-α secretion from activated macrophages via CD200R-vOX2 interaction (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). Viral homologs to CD200 span large taxonomical gaps, including 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). These evidences suggest a common viral immunoevasion strategy which negatively restricts activated macrophages, thus potentially providing an explanation for KS sarcomagenesis (Chung et al., 2002; Foster-Cuevas et al., 2004). vOX2 fused with the Fc domain of human immunoglobulin G1 has been reported to inhibit neutrophil oxidative burst and inhibited the production of pro-inflammatory chemokines (IL-8 and monocyte chemo-attractant protein 1) by monocytes/macrophages. Therefore, vOX2 may be associated with immune dysfunction and could have anti-inflammatory therapeutic potential (Rezaee et al., 2005).

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CHAPTER II

MOLECULAR DETERMINANTS OF KSHV TUMORIGENICITY

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), was first discovered in 1994 in Kaposi's sarcoma (KS) tumors of AIDS patients. KSHV is a member of the gamma-2-herpesvirus family (genus *Rhadinovirus*) (Neipel et al., 1997; Russo et al., 1996) and associated with the development of Kaposi's sarcoma, primary effusion lymphoma (PEL) or body cavity-based lymphoma (BCBL), and multicentric Castleman's disease (Antman and Chang, 2000; Ganem, 1998; Schulz, 1998a; Schulz, Sheldon, and Greensill, 2002). KSHV infects many human cell types; furthermore, KSHV has a number of oncogenes which alter target cell functions. Envelope-associated glycoprotein B gene is encoded by one of these oncogenes and is the focus of this chapter (Sarid et al., 1998).

Herpesviruses, as a general rule, initiate viral attachment to the cell surface by binding to ubiquitous glycosaminoglycans, such as heparan sulfate-like (HS-like) molecules. This attachment is mediated by several envelope-associated glycoproteins. Earlier studies have shown that KSHV binds to host cell HS-like receptors via the K8.1 protein and the RGD motif of gB. In contrast to the gB protein of EBV, KSHV gB is expressed on the surface of the cells and on the virion envelope (Akula et al., 2001a; Pertel, 2002; Wang et al., 2003). In addition, a recent study indicates that gB can regulate many cellular signaling pathways and facilitate virus penetration into host cells by specifically binding to the α5β3 integrin through an RGD (Arg-Gly-Asp) motif. gB-mediated signaling pathways and cytoskeletal remodeling play important roles in the development of tumors (Sharma-Walia et al., 2004). Viral glycoprotein B is also

important in the fusion of the KSHV viral envelope with either cellular or endosomal membranes, as well as virion morphogenesis and egress (Birkmann et al., 2001).

The mechanisms of KSHV pathogenesis and tumorigenesis have not been fully elucidated due to the lack of appropriate animal models susceptible to KSHV infection. Since KSHV can only replicate in human cells, severe combined immunodeficient (SCID) mice have been engrafted with normal human tissue and subsequently injected with purified KSHV virions (Dittmer et al., 1999; Foreman et al., 2001; Picchio et al., 1997). Alternatively, KSHV-infected human tumor cells, such as PEL, have also been injected into SCID or nude mice (Komanduri et al., 1996; Salahuddin and Markham, 1988; Zenger et al., 2002). Mice injected with the cell-free KSHV virus did not develop tumors or clinical symptoms. However, ascites tumors were observed in SCID mice injected with KSHV-positive BCBL-1 cells. Studies on different BCBL-1 cell injection sites have suggested that intraperitoneal or subcutaneous injections of the PEL cell line in mice cause them to develop ascites or solid tumors (Picchio et. al., 1997). Moreover, the extracellular matrix is important for the development of tumors, and Matrigel matrix (solubilized basement membrane) is believed to facilitate PEL tumor growth (Staudt et al., 2004). A recent study shows that primary effusion lymphomas in NOD/SCID mice are inhibited by a combination of azidothymidine and interferon-alpha (Wu et al., 2005). Long-term-infected telomerase-immortalized endothelial (TIVE) cells have been successfully developed as a model for evaluation of KSHV latency *in vitro* and *in vivo* (Grisotto et al., 2006).

Stable 100% KSHV infected PEL cell lines are the most consistent model to study KSHV gene expression and function as well as the effects of infection. Many previous studies of human cancer cell types have suggested that the implantation of cells in nude mice have similar biological behaviors to these cancer cells in humans. An effective example of this is human

prostate cancer cells which form primary tumors but metastasize inefficiently when implanted in nude mice.

The overall objective of this study was to develop an animal model that could be used to assess the potential molecular determinants of KSHV tumorigenicity *in vivo*. RNA interference has been used as a powerful tool for silencing the function of specific genes (Arens et al., 2005; D'Alessio et al., 2004; Schweinitz et al., 2004). siRNAs provide a great potential strategy not only for the examination of gene function but also for the development of gene-specific therapeutic modalities (Gondi et al., 2003; Jiang, Rubbi, and Milner, 2004; Miyagishi and Taira, 2003). Previous studies in our laboratory have shown that the KSHV gB is important in virion egress from BCBL-1 cells. With this in mind, we injected PEL or BCBL-1 cells transfected with plasmid vectors expressing anti-gB siRNA and codon-optimized gB that cannot be recognized by anti-gB siRNAs in the presence of Matrigel subcutaneously in nude mice. Tumors treated with anti-gB siRNAs were significantly smaller than those in codon-optimized gB and negative control groups. The results of this study indicate that gB plays important roles in KSHV-induced tumorigenicity.

MATERIALS AND METHODS

Cells and Viruses Propagation

The BCBL-1 cells harboring the rKSHV.152 genome constitutively expressing the green fluorescent protein (gift from Dr. Vieiria) were routinely grown in RPMI 1640 medium (HYCLONE) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HYCLONE), 2mM glutamine, 1% penicillin/streptomycin (pen/strep) (GIBCO) and 250 mg of G418/ml (GIBCO). The rKSHV.152 contained the green fluorescence protein (GFP) gene cassette under the human cytomegalovirus (CMV) immediate-early promoter, constitutively expressing the

GFP gene. 293 A cells (American Type Culture Collection, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% FBS, 2 mM L-glutamine and 1% pen/strep. Cell cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2. The KSHV lytic cycle was induced in the BCBL-1 cells by adding 25 ng/ml of 12-O-tetracanoylphorbol-13-acetate (TPA, Sigma, St. Louis, Missouri). Virus from the supernatant was collected after 16 hours and treated with DNase I before extraction of viral DNA as described previously (Luna et al., 2004). The supernatants were collected 48 hours post induction with TPA for infectivity studies.

Antibodies

Rabbit polyclonal antibodies generated in our laboratory against KSHV gB peptides were used to detect gB protein in western blot analysis as described previously (Baghian et al., 2000). The 16 peptide antibodies specific to 167-191 amino acids (a.a) of the gB protein were used in this study. Mouse anti-Hu GAPDH monoclonal antibodies were used to detect the GAPDH. Other antibodies were used in this study including rat polyclonal antibodies to LANA, mouse monoclonal antibodies to K8.1, mouse monoclonal antibodies to ORF 59 (Advanced Biotechnologies, Inc., Columbia, Md).

Vectors Construction

The siRNAs were constructed to target the 5' region of KSHV gB expression sequence. This construction was accomplished using AMBION's online siRNA Target Finder. The siRNA 18 was located from the nucleotide 403 after the ATG codon, while siRNA 22 located starting at 484 bases from the ATG codon. Two siRNAs specifically selected against gB were generated as follows: si18 (sense strand HindIII---5`AGCTTCAAGTATGAACTCCCGAGATTCAAGAGA TCTCGGGAGTTCATACTTGTTG-3`; anti-sense strand-EcoRI--5'AATTCAACAAGTATG

AACTCCCGAGATCTCTTGAATCTCGGGAGTTCATACTTGA-3'); si22 (sense strand-HindIII—5'AGCTTGGTAAATGTCAACGGGGTATTCAAGAGATACCCCGTTGACATTT ACCTTG-3'; anti-sense strand-EcoRI---5'AATTCAGGTAAATGTCAACGGGGTATCTCTT GAATACCCCGTTGACATTTACCA-3'). The siRNAs oligonucleotides containing HindIII and EcoRI were designed to carry the HindIII and EcoRI overhangs at the 5' and 3' ends, respectively.

The siRNA 18 and siRNA 22 were denatured at 94 ºC for 5 minutes and cool down slowly to room temperature allowing for annealing of the two strands. Each annealed siRNAs was cloned into the HindIII / EcoRI digested p3xFLAG vector by T4 DNA ligase (NEB). The ligation mixture was transformed into One Shot TOP10 chemically competent E. coli cells (Invitrogen) and spread on LB agar plate containing 100ug/ml ampicillin. All recombinant constructs were confirmed by further restriction enzymes digestion and sequenced to their structure.

Codon Optimization

The codon optimization of the first 540 bases of KSHV gB, from ATG to the HpaI restriction site, were accomplished by using the DNAWorks Web Site: http://molbio.info.nih.gov/dnaworks. This codon-optimized fragment was designed to replace the first 540 bp region of WT KSHV gB carrying siRNA's targeting sequences between the KpnI and HpaI sites. The codon-optimized gB was cloned in the expression vector p3xFLAG between the KpnI and BamHI sites. The wild type KSHV gB was cloned in the expression vector p3xFLAG within the KpnI and BamHI sites. A 577bp ECMV internal ribosome entry site (IRES) was amplified from pIRESHyg3 vector (Clontech) by using the primers indicated in Table 1. The recovered IRES fragment inserted between the BglII and EcoRV restrictions sites

was placed downstream of the siRNA and upstream of codon-optimized gB in the p3xFLAG vector.

Transient Transfection of BCBL-1 Cells

Transient transfection of BCBL-1 cells was carried out using Superfect (Qiagen) transfection agent according to the manufactuere's instructions. BCBL-1 cells were counted, followed by 3 washes in phosphate-buffered saline (PBS). 0.5 x 10^5/well cells were plated in 12-well plates. Cells were transfected with plasmids containing si18 IRES and si22 IRES together, p3xFLAG vector (negative control plasmid), and codon-optimized gB siRNA IRES mixed with Superfect in PRMI 1640 as recommended by the manufacturer. All transfections contained the same amount of total transfected DNA. After 4 h of incubation at 37 ºC, fresh PRMI 1640 with 10 % fetal bovine serum was added.

Tumor Formation

Cells were counted, washed once in ice-cold PBS, and diluted in cold phenol red-free Matrigel (BD biosciences discovery labware). Female athymic nude mice (4 weeks of age; Charles River Laboratories) were housed in autoclaved cages with high-efficiency filter tops and autoclaved bedding. The animal room was kept at 25 ºC with a 12-hr-dark cycle. At 6-7 weeks of age, the nude mice (n=10/group, 19-21g body weight) were injected subcutaneously with 5 x 10^6 transient transfected BCBL-1 cells containing 200 ul Matrigel. The mice were then observed every day for palpable tumors. Body weights were determined weekly, and tumor size was measured. Measurements were taken after 7 days of tumor inoculation using a digital microcaliper. Tumor volumes were calculated twice a week according to the following formula: volume = (length x width x height)/2. Subcutaneous masses with the overlying skin as well as the spleen, liver, kidney, heart, and lung removed from the nude mice were fixed in 10% neutral

formalin and stained with Hematoxylin and Eosin (H&E) stain (Vector Laboratories). Unstained slides from the above subcutaneous masses were routinely analyzed by using immunofluorescence assays.

The animal studies were approved by the Institution Animal Care and Use Committee (IACUC) of Louisiana State University (Baton Rouge). Animal care was in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, 1996).

***In vivo* Imaging**

Mice were anesthetized through the use of intraperitoneal injections of Avertin (2-2-2 Tribromoethanol) at 0.2-0.4 mg/g. Three min after Avertin taking effect, the mice were transported and imaged for 5-7 min using KODAK In-Vivo Imaging System FX Pro (Carestream health). This system provides molecular signal localization in live animals.

Viral DNA Preparation

Tumors from mice were prepared in PBS and homogenized manually with an all-glass Potter Elvehiem homogenizer (KIMBLE / KONTES.). TurboDNase I (Ambion) were used to treat 200 ml of the tumor homogenates for 2 hours at 37 ºC to ensure that nonencapsidated genomes were absent. In these experiments, 1 U of TurboDNase I was used per 100 ul of sample. Viral DNA from tumor homogenates was extracted by using the DNeasy blood and tissue Kit (Qiagen) as the manufacturer's instructions.

Immunofluorescence Assay

Antigen retrieval was done by microwaving tissue in 1.5% antigen unmasking solution (Vector Laboratories) for 20 min at 60 ºC and cooling at room temperature for 20 min. The 4µm sections from formalin-fixed and paraffin-embedded tumor tissues were washed with 1x PBS (phosphate

buffered saline) and 1x PBS containing 0.1% gelatin from cold water fish skin (FSG) (Sigma-Aldrich) for 10 min, respectively. The tumor sections were blocked by 10% mouse serum for 15 min followed by 200 ul of DakoCytomation protein-block-serum-free solution (DakoCytomation) of each slide for 15 min at room temperature. The sections were first stained with rat polyclonal antibodies to LANA, mouse monoclonal antibodies to K8.1, mouse monoclonal antibodies to ORF59 (Advanced Biotechnologies) at 1:100 dilution and rabbit anti-gB polyclonal antibodies at 1:500 dilution for 1 h at room temperature in the humidity box. Slides were washed with 1x PBS/FSG for 10 min and then incubated with AlexaFluor 488-labeled goat anti-rat IgG, AlexaFluor 488-labeled goat anti-mouse IgG, or AlexaFluor 488-labeled goat anti-rabbit IgG (Invitrogen) for 30 min at room temperature in the humidity box. All AlexaFluor 488-labled goat IgGs were filtered with 0.2um PES w/GMF (Whatman) filters before being applied to slides. All the slides were washed once with 1x PBS and the nuclei were stained by incubation with 1ug/ml of DAPI (Invitrogen) for 30 min at room temperature. Slides covered with cover-slips were washed once with 1x PBS and observed using fluorescence microscopy.

BCBL-1 Cells Proliferation Rate Assay

The *in vitro* cell proliferation rate of transiently transfected and non-transfected GFP-positive BCBL-1 cells was determined by plating BCBL-1 cells (in triplicate) with and without Matrigel in the wells of 96-well plates. BCBL-1 cells were transfected with plasmids containing siRNAs, siRNA-codon-optimized gB and empty vector p3xFlag. Transfected and non-transfected GFP-positive BCBL-1 cells plated in the absence of Matrigel in the other 96-well plate were used as controls. Cells were harvested and counted using a hemocytometer from day 1 to day 7.

Statistical Analysis

The SAS® (Version 9.1.3) GLM procedure was used to analyze the data of tumor volumes in an analysis of variance of a split-plot arrangement. Statistical analyses using this model included the treatment group and the group of untreated animals as main plot effects, and the Period and Treatment Group by Period interaction as subplot effects. When overall F tests indicated significance, post hoc pairwise comparisons of main effects were conducted with Turkey's HSD test. Pairwise comparisons of significant interaction effects were made with t tests of least-square means. All comparisons were considered significant at $P \leq 0.05$.

Table2.1: Synthetic Oligonucleotide Primers

PRIMER NAME	SEQUENCE 5\`-3\`
Ires For.	agatctcactagaggaattccg
Ires Rev.	gatatcgtggcaagcttatcatc
gB Universal For	tccagactacccacgaggac
gB Universal Rev	gtcaggttaatcgcggacat
gB WT For	gacacctttcagacgt
gB WT Rev	tctcgggagttcatacttgt
Orf 59 Pro	FAM cgcgtgagctattcggtgcgaata TAMRA
Orf 59 For	tcagcttcaggaatacgtccg
Orf 59 Rev	ggctatgccagcgtcgagta
GAPDH For	gattccacccatggcaaatt
GAPDH Rev	aagatggtgatgggatttccatt

RESULTS

Construction and Characteristics of Expression Plasmids

To study the role of glycoprotein B in KSHV virus egress and tumor progression, we used siRNAs to knockdown gB gene expression in BCBL-1 or PEL cells. gB expression has been linked to virus-induced cell fusion as well as entry. First, we developed p3xFlag-CMV plasmid vectors containing the wild-type (WT) gB genes and codon-optimized gB. The corresponding WT region, from ATG to the HpaI site of the codon-optimized gB, was replaced with a 540 base fragment amplified by a set of 22 overlapping primers. siRNAs-IRES driven by human CMV promoter, were cloned by inserting siRNAs between the HindIII and EcoRI sites followed by an IRES fragment. The 577 bp internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) was amplified from pIRESHyg3 vector, which allows the expression of multiple proteins from one transcriptional mRNA. The consistent expression of siRNAs targeted gB at the positions of 403 bp and 483 bp respectively. siRNAs-IRES-codon-optimized gBs were constructed by inserting the IRES fragment downstream from between siRNAs and codon-optimized gB sequentially into the p3xFlag-CMV vector. Next, siRNAs-IRES plasmids and siRNAs-IRES-codon-optimized gBs were transfected in the 293 cells and BCBL-1 cells. BCBL-1 cells transfected with Superfect, wide type gB and non-specific siRNAs were used as controls.

Transient Expression of gB In 293 Cells

As shown in Figure 2.3, analysis of the siRNA-transfected 293 cells for gB gene expression via reverse transcription-PCR demonstrated a significant reduction in mRNA levels of gB in 293 cells. However, gB expression was recovered in siRNA-IRES-codon-optimized gB transfected cells. In contrast, there was no dramatic inhibition of gB expression in 293 cells alone, Superfect-transfected 293 cells, wide type gB-expressing vector transfected 293 cells, or non-

specific siRNAs plasmid transfected 293 cells. Western blot analysis of cell debris showed that decreased gB protein production (as indicated in Figure 2.3) was associated with decreased mRNA gB expression. Similarly, no effects of inhibition of siRNAs were observed on the expression of GAPDH, which was used as an internal control for specificity and loading mRNA

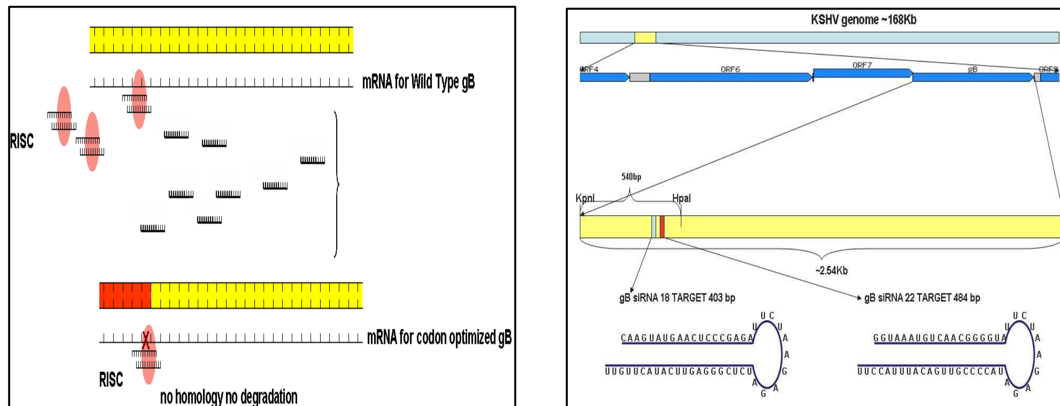


Figure 2.1: Small Interfering RNAs (siRNAs) Selectively Block Expression of Genes. siRNAs binds to the RNAi silencing complex (RISC) which first mediates unwinding of siRNAs and binds to its target mRNA in a sequence specific way. Codon-optimized gB can't be degraded since the target sequence of siRNAs is replaced by the codon-optimized sequence. siRNAs oligonucleotides were synthesized to target WT gB at the positions 403 bp and 484 bp.

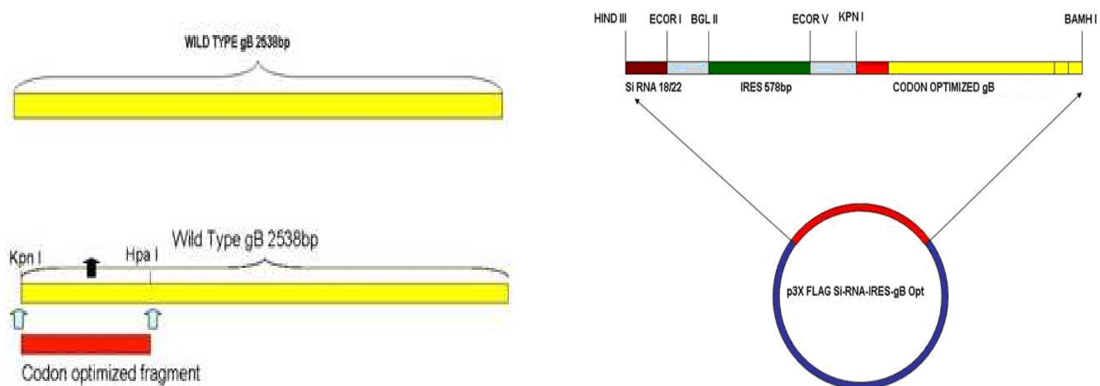


Figure 2.2: Strategy Used to Construct the Codon-Optimized KSHV gB. The codon-optimized fragment amplified by PCR replaced the WT gB from the first 540 base pairs between the restriction sites KpnI and HpaI. siRNA-IRES-codon-optimized gB was cloned into p3xFlag with siRNAs insertion between the HindIII site and EcoRI site followed by codon-optimized gB. The IRES gene was inserted at the restriction sites BglII and EcoRV between siRNAs and codon-optimized gB.

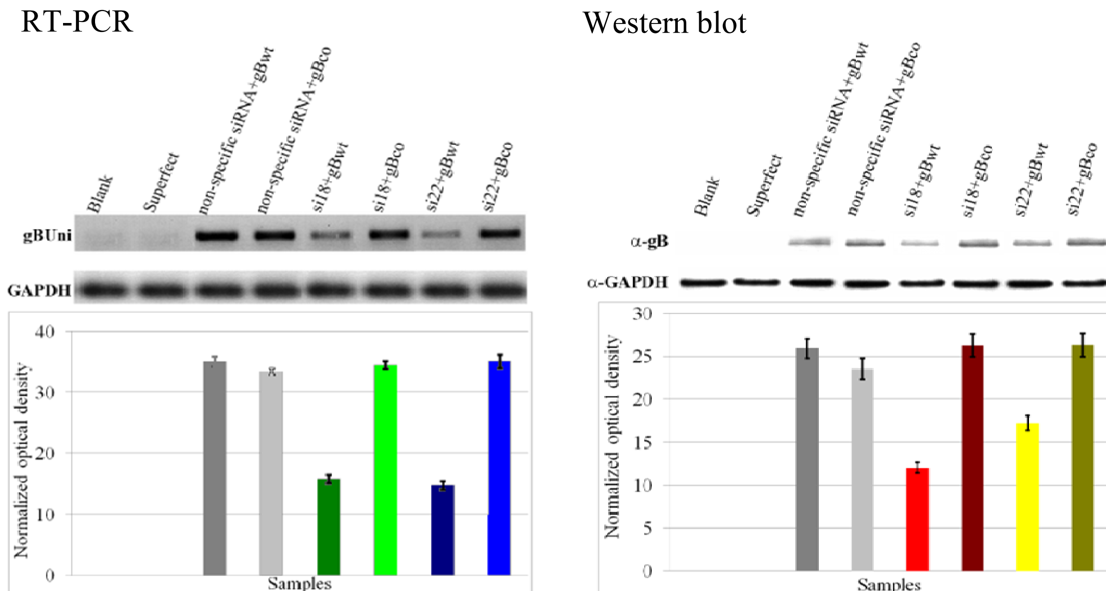


Figure 2.3: siRNAs 18 and siRNAs 22 Specifically Inhibit KSHV gB Expression in 293 Cells. Mock and 293 cells transfected with siRNAs and codon-optimized gB plasmids were examined by Reverse Transcription-PCR. Total RNA was extracted from mock and 293 transfected cells as described in materials and methods. The GAPDH mRNA was also amplified as an internal control. Western blot of total protein extracted lysates was extracted from cell debris. GAPDH was used as a loading control. siRNAs specifically down-regulated KSHV gB expression at both mRNA and protein levels.

levels as well as protein levels. These investigations clearly demonstrated the following: Wide type gB effectively complimented codon-optimized gB in 293 cells. More importantly, siRNAs 18 and siRNAs 22 specifically inhibited KSHV gB expression.

Transient Expression of gB In BCBL-1 Cells

BCBL-1 or PEL cell lines have been shown to represent the most consistent model for studying KSHV gene expression and function in tumorigenicity. KSHV expresses a small percentage of lytic infected cells while mainly expressing latent cells, which can be induced to become lytic with the 12-O-tetradecanoylphorbol 13-acetate (TPA). Analysis of the effects of gene-specific siRNAs 18 and siRNAs 22 on KSHV gB expression in BCBL-1 cells used reverse transcription-PCR. Transient transfected BCBL-1 cells were induced by 25ng/ml TPA for 12 hours and

harvested. Total soluble RNA was extracted from cellular debris treated with DNase I. The mRNA level of WT gB expression was drastically reduced in siRNAs transfected BCBL-1 cells; while no significant inhibition of WT mRNA gB expression was observed in either siRNA-IRES-codon-optimized gB transfected cells or control BCBL-1 cells. Similarly again, reduced gB protein expression was correlated with siRNAs transfected cells in contrast to siRNA-IRES-codon-optimized gB transfected or control BCBL-1 cells. The expression of GAPDH was not inhibited by siRNAs, which was used as an internal control to normalize gB mRNA levels as well as protein levels. Therefore, siRNAs 18 and siRNA 22 effectively silenced KSHV gB mRNA levels as well as protein expression in both the 293 cells and BCBL-1 cells.

Quantification of KSHV by TaqMan PCR

To better quantify the amount of virus produced by the transfection of BCBL-1 cells, a real-time PCR assay targeting ORF59 (a KSHV early lytic gene) was essential. KSHV ORF59 encodes a viral DNA polymerase processivity factor involved in viral DNA replication. Viral DNA was extracted using DNeasy tissue and Blood kit in the above supernatants of transfected BCBL-1 cells. KSHV DNA was generated based on known amounts of BAC36 DNA containing the WT KSHV genome. The concentration of purified BAC36 DNA was determined by measuring optical density at 260 nm and by comparative gel electrophoresis using known amounts of molecular markers. A standard curve based on the C_t value and logarithmic amounts of diluted BAC36 was constructed by diluting BAC36 DNA at serial 10-fold levels. The C_t value was identified as the cycle number at which fluorescence detection exceeded an established constant threshold level among all the experiments.

As shown in Figure 2.5, siRNA 18- and siRNA 22- transfected BCBL-1 cells produced relatively large numbers of KSHV genomes in supernatants, with a little higher amount of viral

genomes produced in the siRNA 18 group. BCBL-1 cells alone or transfected with Superfect produced about two-fold more viral KSHV genome than cells transfected with siRNAs18 and

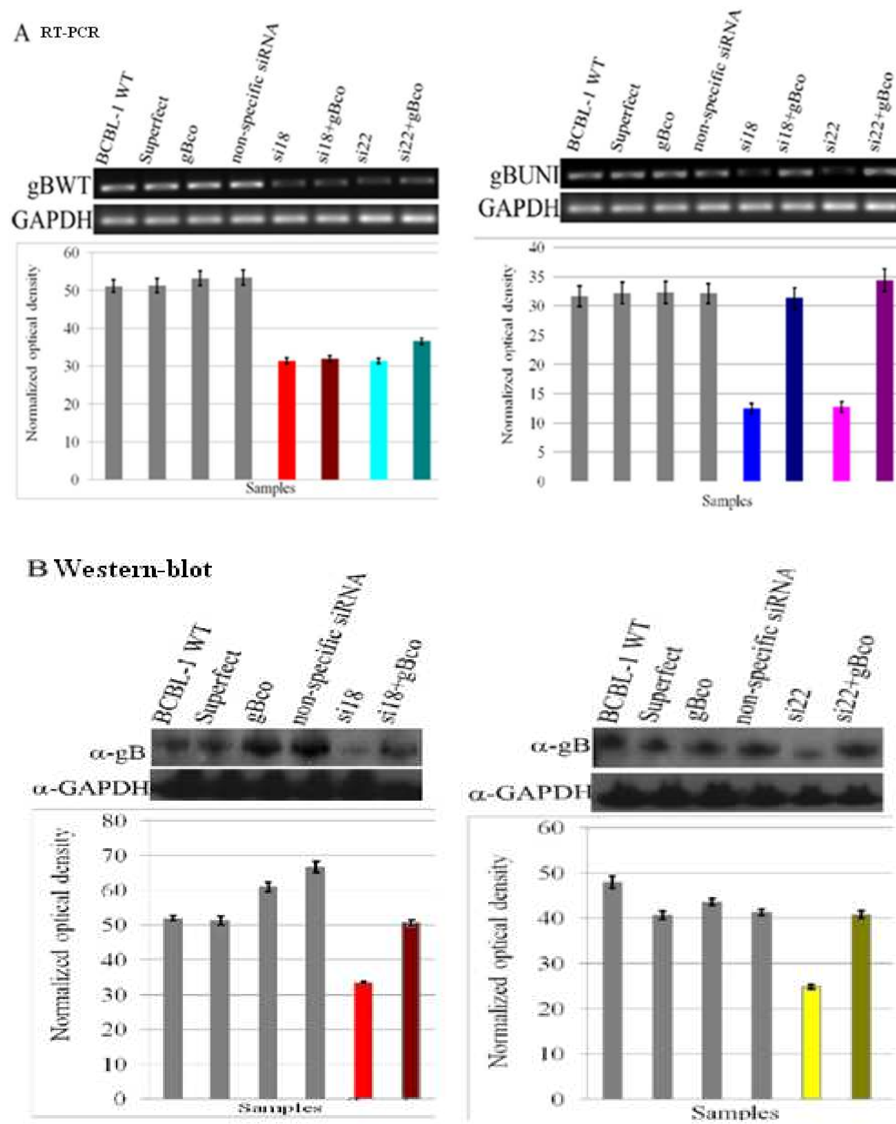


Figure 2.4: siRNAs 18 and siRNAs 22 Specifically Inhibit KSHV gB Expression in BCBL-1 Cells. Reverse Transcription-PCR was used to examine gB expression in mock and BCBL-1 cells transfected with siRNAs and codon-optimized gB plasmids. Total RNA was extracted from mock and transiently transfected BCBL-1 cells as described in materials and methods. The GAPDH mRNA was an internal control. Western blot of total protein extracted lysates was extracted from cell debris. The GAPDH protein was used as a loading control. siRNAs specifically inhibited KSHV gB expression at both mRNA and protein levels in BCBL-1 cells.

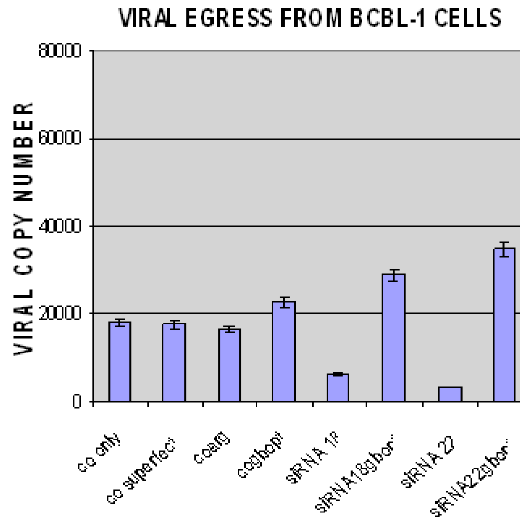


Figure 2.5: Reduced KSHV Genomes in Supernatants from BCBL-1 Cells Transfected with siRNAs Determined by Quantitative Real-time PCR. Supernatants were treated with DNase I to remove nonencapsidated genomes. Viral DNA and real-time PCR used the supernatants from cells transfected with plasmids containing siRNAs and siRNAs-IRES-codon-optimization gB. Supernatants from cells transfected with non-specific siRNAs, Superfect and mock cells were used as controls. Error bars indicate standard deviations.

siRNAs 22. This effect of inhibition was siRNAs specific, since transfection of non-specific siRNAs did not down-regulate the expression of viral genomes found in supernatants.

Codon-optimization has been indicated as a powerful tool for enhancing protein expression by altering gene sequences from small RNA and DNA viruses (Bradel-Tretheway, Zhen, and Dewhurst, 2003; Villalobos et al., 2006). Hence, the number of KSHV genomes found in supernatants of codon-optimized gB transfected BCBL-1 cells were a little higher than in Superfect, non-specific siRNAs transfected and negative control BCBL-1 cells; this may be due to the codon optimization in the gB gene sequence. Transfection of siRNAs 18-codon-optimized plasmid yielded approximately three fold more viral genomes in BCBL-1 cells compared to those found in siRNAs transfected BCBL-1 cells.

Characteristics of PELs in Nude Mice

In our studies, BCBL-1 cells were transiently transfected with the same plasmids as mentioned above. Female athymic nude mice (nu/nu; n=10/group) were injected subcutaneously in the interscapular area with $5x10^6$ transiently transfected BCBL-1 cells mixed with Matrigel. Tumor incidence and tumor volume were recorded twice a week until sacrifice. Mice weight was measured once a week. The palpable tumor was typically observed 7 and 14 days after tumor inoculation. When the tumor growth was detectable, the tumor volume (mm^3) was measured every 3-4 days. Representative figures of tumors in mice injected with transfected BCBL-1 cells are presented in Figure 2.6.

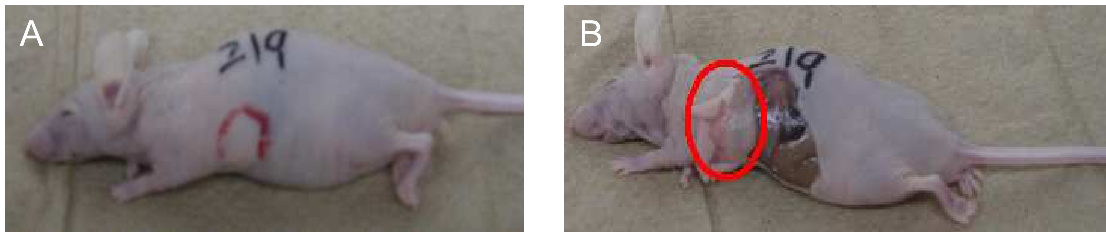


Figure 2.6: A Representative Subcutaneous Mass before and after Dissection. A. Mouse showing subcutaneous PEL tumor in the presence of Matrigel four weeks after inoculation. B. Appearance of PEL tumor in Matrigel after dissection of the skin (the red circle).

In Vivo Imaging of PEL Tumors

As shown in Figure 2.7, BCBL-1 cells constitutively expressed the GFP gene, allowing for detection of tumor progression and eukaryotic cells containing KSHV genomes under fluorescence microscopy. Constitutive expression of GFP was produced by the KSHV genomes that containing green fluorescence protein (GFP) gene cassette driven by the human cytomegalovirus (CMV) immediate-early promoter. KSHV is essential for the development of primary effusion lymphomas. In order to localize KSHV genomes in subcutaneous tumors in live mice, we use X-ray and fluorescence to image living nude mice. This is an example of the

tumor in nude mice which contains the GFP-positive tumor by X-ray and florescence as shown by Figure 2.7.

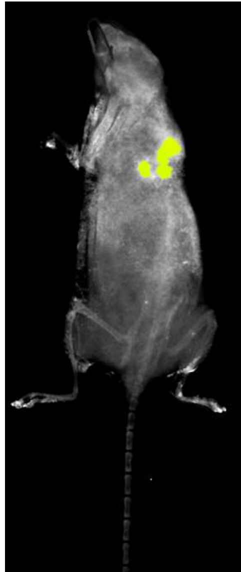
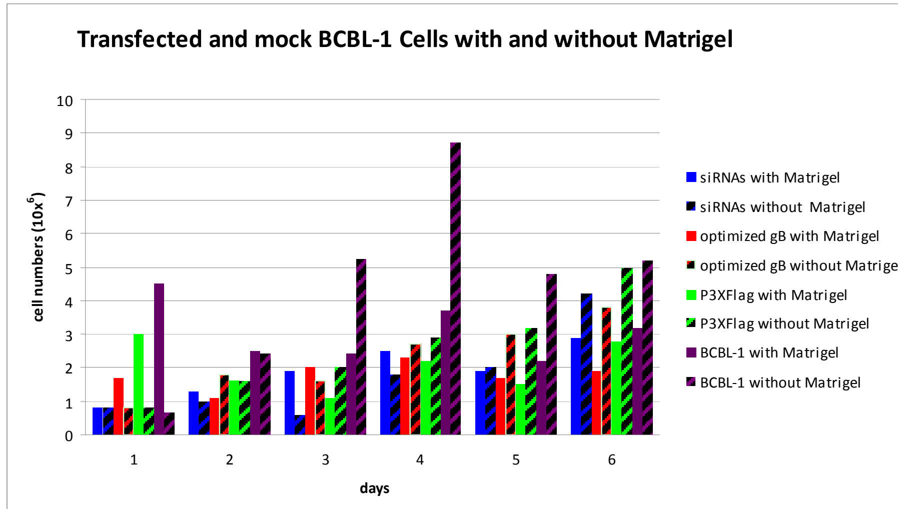
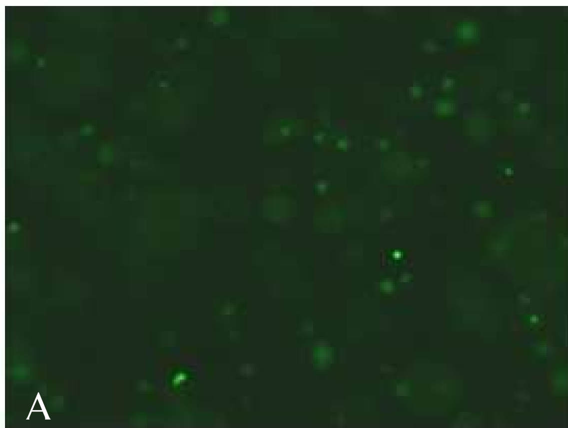


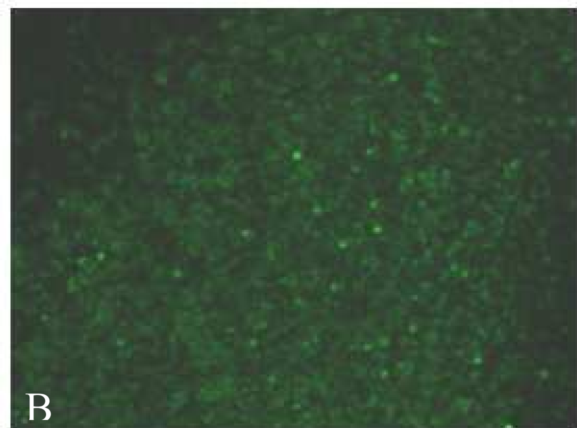
Figure 2.7: X-ray and Fluorescent Image of Nude Mouse . GFP-expressing BCBL-1 cells were injected into the interscapular area of nude mice. Mice were anesthetized with Avertin i.p. and images were taken by using KODAK In-Vivo Imaging System FX Pro. Detection of green fluorescence from GFP gene in cells indicated KSHV genomes in PEL tumors.

Proliferation of BCBL-1 Cells *In Vitro* with Matrigel

The RGD motif of gB is important for cell attachment via the interaction between cell surface molecules and integrins. In addition, the RGD sequence participates in vascular development and remodeling mediated by binding to extracellular matrix (ECM) proteins and endothelial cells. Transiently transfected BCBL-1 cells were plated in 96-well plates in either the presence or absence of Matrigel. Cell numbers were counted every day, from day 1 to day 6. As shown in Figure 2.8B, GFP expressing BCBL-1 cells tend to grow together in the center of wells in contrast to the distinct distribution of individual cells in the matrigel. In the absence of matrigel, BCBL-1 cells transfected with plasmids containing siRNAs, siRNAs-IRES-codon-optimized gB and negative control p3xFlag vectors grew faster higher than cells supported by Matrigel (graph). No significant differences were observed among different groups of BCBL-1 cells.







Cells with Matrigel

Cells without Matrigel

Figure 2.8: *In Vitro* GFP+ BCBL-1 Cells Proliferation Rate with matrigel. Cells were counted every day by using hemocytometer. GFP expressing BCBL-1 cells in the absence of Matrigel were tended to grow together in the center of the well. In contrast, in the presence of Matrigel, cells grew separately. All images were at x50 magnification level.

PEL Tumor Growth

Subcutaneous masses developed in nude mice injected with siRNAs transfected BCBL-1 cells produced a highly significantly smaller size of tumors ($P < 0.0001$) in comparison with controls of codon-optimized, negative control p3xFlag vectors and mock cells alone 17 days after inoculation (Figure 2.9). Tumors with Matrigel formed in nude mice injected with codon-optimized transfected PEL cells showed no significant difference from those obtained from non-transfected BCBL-1 cells with slightly larger tumor volumes. Statistical analysis indicated that

tumors injected with negative control p3xFlag vectors were significantly different from that injected with codon-optimized, siRNAs transfected or non-transfected mock cells ($p<0.0001$). From day 7 to 30 after inoculation, tumors in mice injected with codon-optimized, p3xFlag vectors and BCBL-1 cells were not significantly different within the same periods as indicated by the overlapped bars. Correlation analysis of mice weight and tumor growth demonstrated that

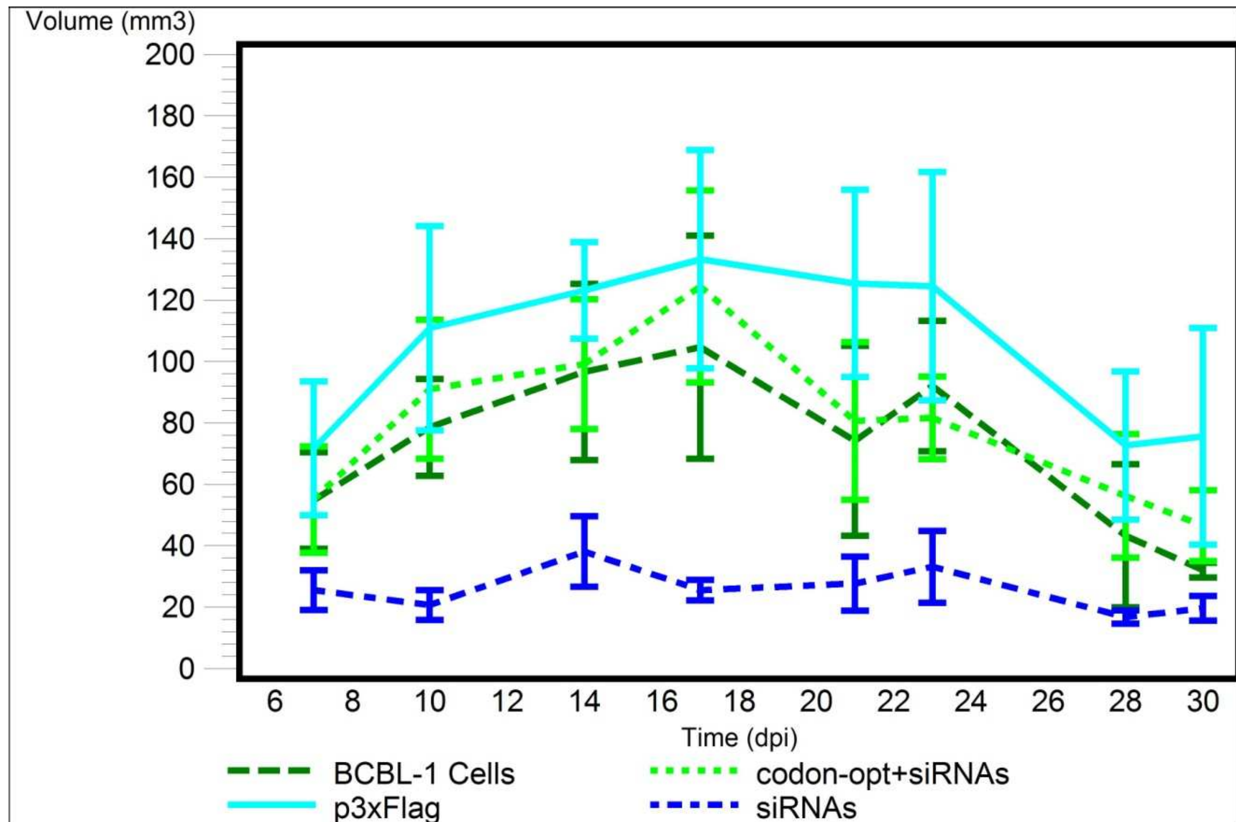


Figure 2.9: Effect of Transfected BCBL-1 Cells in Nude Mice. Primary effusion lymphomas were established by injecting subcutaneously mock-transfected and transfected BCBL-1 cells with various plasmids in the presence of Matrigel into the interscapular areas of nude mice. Tumor volumes were recorded with a digital caliper every 3-4 days (*x* axis). BCBL-1 cells were transiently transfected with plasmids harboring siRNAs-IRES, siRNAs-IRES-codon-optimized gB and p3xFlag vector (negative control). "0" indicates the first day of injection. Tumor volumes were estimated according to the formula: volume = (length x width x height)/2. All results at each time points are presented as means $\pm$ standard errors. Overlapped standard error bars indicated a not significant difference among different groups.

tumor growth was not affected by different weight gain/loss in all four study groups of nude mice injected with cells transfected different plasmids (data not shown).

Pathobiological Examination of Tumors

Neoplastic cells were observed within the subcutaneous masses; however, there was no evidence of metastasis in spleen, liver, kidney, heart, or lung. No overt histological difference was present in the subcutaneous masses among the groups. The masses were composed of multiple islands and/or sheets of round to polygonal neoplastic cells admixed with abundant eosinophilic homogeneous material (Matrigel) (Figure 2.10A). The sheets of neoplastic cells were mostly present at the periphery of the masses. The neoplastic cells were shown to have a round to irregular shaped nuclei with small to moderate amount of cytoplasm (Figure 2.10B). Based on the nuclear morphology, the neoplastic cells were separated to three types: 1. lymphoblastic cell type characterized by a large irregular shaped hyperchromatic nucleus with an obscured nucleoli; 2. immunoblastic cell type characterized by a large irregular shaped nucleus with single or multiple prominent nucleoli; 3. small lymphocytic cell type characterized by a small round hyperchromatic nucleus with an obscured nucleolus. Each neoplasm consisted of single or mixed cell types described above. The mitotic rate was 0 to 15 per high power field (x400), and there were occasional binucleated cells. Occasionally, neoplastic cells were separated by clear spaces, which were most consistent with an artifact during the process of sectioning. Multifocal necrosis was present within all masses except one injected BCBL cells. There were rare infiltrates of macrophages. All masses contained small numbers of blood vessels, which were lined by plump endothelial cells and contained erythrocytes (Figure 2.10B), and/or spindle cells interpreted as endothelial cells forming capillaries or fibroblasts (Figure 2.10B inset).

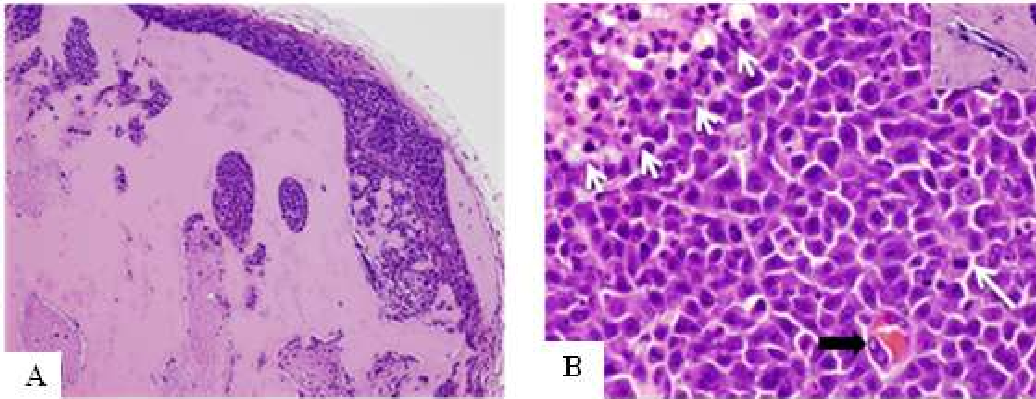


Figure 2.10: Subcutaneous Masses Stained with Hematoxylin and Eosin (H&E). (A) Multiple islands and sheets of neoplastic cells were observed within abundant eosinophilic homogeneous material (Matrigel) as well as multifocal necrotic areas. (B) Round to polygonal neoplastic cells had large irregular shaped nuclei containing occasional prominent nucleoli, with multiple mitotic figures (long white arrow). A focal area of necrosis characterized by pyknotic nuclei and cellular debris was surrounded by short white arrows. The blood vessel lined by plump endothelial cells and filled with erythrocytes was indicated by a black arrow. Spindle cells within Matrigel were shown inset.

Characterization of KSHV Infected PEL Tumor Cells

Previous studies have indicated that the marker of KSHV latency, latent nuclear-associated (LANA) protein, is expressed in all the PEL or BCBL-1 cells in suspension culture. To further determine the presence of KSHV in the GFP^+ BCBL-1 cells of tumors; immunofluorescence was carried out to examine the expression of the LANA antigen of PEL tumors supported by Matrigel in mice. As seen in Figure 2.11D, most of the tumor cells were positively stained for LANA in tumors derived from BCBL-1 cells. The LANA antigen was detected as a classic nuclear punctuated pattern suggesting KSHV episomes in the host chromosome (Ballestas, Chatis, and Kaye, 1999) as shown in Figure 2.11E. To further investigate the characterization of the KSHV-harboring BCBL-1 cells in tumors and the status of their KSHV infection, tumor cells were detected from the transient expression of gB and lytic genes K8.1 and ORF59 by indirect immunofluorescence. Antibodies against glycoprotein B and late lytic antigen K8.1 stained cells

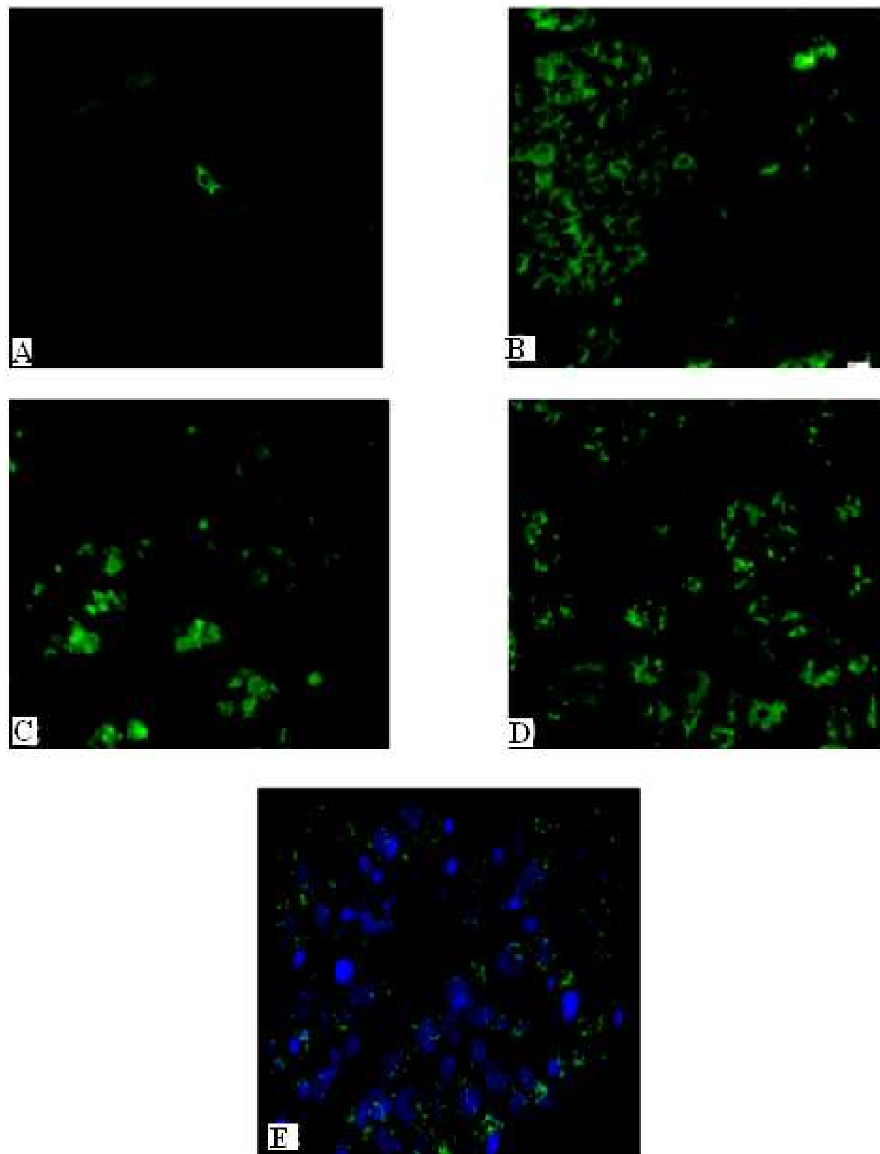


Figure 2.11: KSHV Lytic and Latent Gene Expression of in Matrigel-Supported BCBL-1 Tumors. (A) Tumor tissue was stained with anti-gB rabbit polyclonal antibodies and detected with AlexaFluor 488-conjugated anti-rabbit antibodies in the cell membranes. (B) Tumor sections were stained with mouse monoclonal antibodies against the K8.1 protein and detected with AlexaFluor 488-conjugated anti-mouse antibodies in the cell membranes. (C) The tumor was stained with a mouse monoclonal antibody against the lytic ORF 59 protein followed by secondary antibody, AlexaFluor 488-conjugated anti-mouse antibody. (D) The tumor was stained with a rat polyclonal antibody against the LANA protein and detected with AlexaFluor 488-conjugated anti-rat polyclonal antibodies. (E) Nuclei were stained by DAPI. DPAI is merged with the LANA protein which displays a punctuate pattern. All images are at x 1000 magnification.

were stained in their cell membranes (Figure 2.11 A and B). ORF59 (DNA processivity factor) showed nuclear stain. These results together showed that lytic reactivation from latently infected cells harboring KSHV occurred during the development of tumors.

DISCUSSION

BCBL-1 cell lines harboring patient-derived KSHV have been demonstrated to be the most consistent model for investigating the structure and function of KSHV genes in both lytic and latent replication. Furthermore, virtually all PEL cells express the viral latency-associated nuclear antigen LANA, v-Cyclin, and v-FLIP indicating that KSHV is required for tumorigenesis (Dupin et al., 1999; Fakhari and Dittmer, 2002; Sarid et al., 1998).

RNA interference has been used as a powerful tool for silencing the function of specific genes (Schweinitz et. al., 2004, Arens et. al., 2005, D'Alessio et. al., 2004). Although conventional antisense technologies are sequence selective and provide very good alternatives to conventional gene deletion technologies, it is common for anti-sense RNA to only transiently and partially suppress the expression of the genes of interest (Gondi et. al., 2003, Arens et. al., 2005, Miyagishi et. al., 2003). siRNA provides a new and effective strategy not only for the examination of gene function but also for the development of gene-specific therapeutic candidates (Gondi et al., 2003; Jiang, Rubbi, and Milner, 2004; Miyagishi and Taira, 2003).

The cloning of HHV-8 genome into bacterial artificial chromosome (BAC) allowed the rapid generation of KSHV mutant viruses (Gao, 2003; Zhou, 2002, Zhu 2006). Mutated KSHV viruses have been constructed to investigate the function of KSHV genes essential for growth and replication in tissue culture. However, these viral genomes, in most cases, could not lead to robust infection and production of relatively large numbers of infectious virions effectively limiting their use in investigating the structure and function of individual viral proteins.

In this study, anti-gB siRNAs were used to inhibit gB synthesis in BCBL-1 cells that produce large amounts of infectious virions upon the induction of lytic replication. Co-transfection of BCBL-1 cells with anti-gB siRNAs and the codon-optimized gB, which is not susceptible to siRNA inhibition, effectively increased gB mRNA levels and gB expression. In these experiments, the siRNAs were cloned in tandem with the codon-optimized gB to ensure the co-expression into the same transfected 293 and BCBL-1 cells. gB mRNA expression and gB proteins was reduced by approximately 40-60% in the experiments of transient expression of either si18 or si22 in both 293 and BCBL-1 cells. Therefore, a disproportionate number of BCBL-1 cells may be both transfected and reactivated if the transfection rate of BCBL-1 cells is not associated with lytic gene expression. Recently, BCBL-1 cells in the S phase were shown to be more likely to undergo reactivation and display a smoother surface topology compared to those in G0/G1 phase (Whitman et al., 2007).

Studies have shown that gB may serve both conserved and different functions in gB-mediated cell fusion, virion assembly, egress and infectivity among different herpesviruses. KSHV gB is essential for KSHV egress from 293 cells. These results were obtained using a KSHV gB-null mutant obtained by insertion inactivation of the KSHV gB using a cloned KSHV genome as a bacterial artificial chromosome (Bac) plasmid (Krishnan et al., 2005) . In this study, we showed that siRNA inhibition of gB synthesis led to drastic reduction in virion egress and infectivity in agreement with previous results indicating that gB is essential for virion egress and infectivity. However, the cytoplasmic terminus of gB from amino acid residues 761 to 787 is not essential for either virion egress or infectivity since gB truncations carrying this deletion did not affect virion egress and infectivity. Therefore, these results suggest that KSHV gB domains other than the cytoplasmic domain are involved in virion egress and infectivity. Lack of

gB caused the inhibition of virion egress while complementation of transient gB expression rescued KSHV virion egress from 293 cells. It is possible that either or both the intramembrane and extra-cytoplasmic domains of gB directly or indirectly interact with some membrane bound proteins and glycoproteins associated with virion egress in the cytoplasm.

Our results demonstrated that anti-gB siRNAs quite effectively down-regulated the expression of KSHV gB mRNA as well as protein levels. In order to gain a better understanding of the role of KSHV gB in tumorigenesis, we successfully established a mouse model using anti-gB siRNAs-mediated silencing of gB system. In nude mice subcutaneously injected with BCBL-1 cells transiently transfected with anti-gB siRNAs, we found significant smaller tumors compared with mock- or only codon-optimized gB transfected cells. Tumors growing from mock BCBL-1 in the presence of Matrigel were slightly smaller than those developed in codon-optimized gB transiently transfected BCBL-1 cells supported by Matrigel. This might be due to optimized codon usage that enhances KSHV gB gene expression.

In summary, KSHV glycoprotein B can enhance virus egress from infected cells. There is a slight difference of cell proliferation between transfected and non-transfected BCBL-1 cells in the Matrigel. Statistical analysis of tumor volumes showed that tumors in nude mice injected with siRNAs-transfected BCBL-1 cells were significantly smaller compared to the tumors formed in the mice injected with siRNAs-IRES-codon-optimized gB, negative vector and mock cells. Histological examination demonstrated multiple islands and periphery tumor masses, a number of blood vessels and spindle formation in the tumors with Matrigel. However, differences of neither pathobiological morphologies nor angiogenesis in tumors among different experimental groups were observed. Surprisingly, both latent and lytic genes were detected in tumors by immunofluorescence indicating that KSHV lytic infection may be important in PELs.

What is the reason that tumors were reduced in siRNA-transfected BCBL-1 cells injected nude mice? One possibility is that the production of infectious virus enhances tumorigenesis, possibly by direct infection of adjacent cells including endothelial cells that could enhance angiogenesis and other tumor-assisting functions. Clinical studies show that KSHV lytic replication is critical for KS pathogenesis. In order to expand a tumor mass, latently infected BCBL-1 cells injected into nude mice die and must be replaced by new latently infected cells. To provide such cells, endothelial cells infected with viruses produced by lytic replication become very important. Another possible explanation is that lytic KSHV infected cells may produce some paracrine signaling molecules that lead to promotion in both the inflammation and angiogenesis of PEL tumors. KSHV genome encodes a number of viral genes that produce signaling molecules homologous to cellular cytokines or chemokines. Most of these genes are lytic genes, including viral CC chemokines vMIP, vIL-6, vGPCR (Aoki et al., 2001; Luttichau et al., 2000; Nador et al., 2001). This study may provide new insights into the mechanisms of KSHV tumorigenesis.

Angiogenesis is characterized by the formation of new blood vessels from pre-existing ones. Angiogenesis is a prerequisite for the growth and metastasis of many solid tumors. Angiogenesis is predominantly regulated by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FCG) released by tumor and/or stromal cells. Angiogenesis plays important roles in pathogenesis of hematological malignancies. It was shown that KSHV-infected cells secrete VEGF and induce *in vitro* angiogenesis. KSHV gB has been reported to facilitate KSHV infection and transformation via its interaction with VEGF receptor-3 (Zhang et al., 2005). We also found that anti-gB inhibited the expression of VEGF in BCBL-1 cells transfected by anti-gB siRNAs *in vitro* (Subramanian, personal communication). This may

suggest that anti-gB siRNAs inhibit the interaction between gB and VEGF resulting in the reduction of tumor size in nude mice injected with BCBL-1 cells transiently transfected plasmids coding for anti-gB siRNAs. However, more experiments are needed in order to really understand gB functions.

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CHAPTER III

CONLUDING REMARKS

FUTURE CHALLENGES

KSHV gB is essential for virion egress in 293 cells (Krishnan et al., 2005). Interestingly, we found that the carboxyl terminus of KSHV gB is not essential for virion egress and infectivity (Subramanian et al., 2008). This work suggested that the intramembrane and/or extramembrane of gB may be important for virion egress. Interestingly, HSV-1 gB is known to interact with at least two other viral glycoproteins, gH and gD and these interactions are important for HSV-1 entry into cells and virus-induced cell fusion. Therefore, it is possible that gB interacts with other KSHV viral membrane proteins and glycoproteins that are important in virion egress. Determining the potential role of other KSHV viral glycoproteins and membrane proteins, as well as investigating potential interactions of gB with other viral proteins may shed more light into the regulation of virion egress from infected cells.

Kaposi's sarcoma is thought to be generated after KSHV infection of endothelial cells. Therefore, it is particularly important to investigate KSHV infection of endothelial cells. These studies are very difficult because KSHV does not efficiently infect endothelial cells. In contrast, BCBL-1 cells and other PEL cells produce large amounts of KSHV infectious virus and these cells can form tumors in immunocompromised mice. Using Matrigel to enhance tumor development, anti-gB siRNAs transfected BCBL-1 cells inhibited tumor development in nude mice in comparison to codon-optimized gB, empty vector transfected BCBL-1 cells or alone. This result revealed that KSHV gB is important in the development of tumors. BCBL-1 cells are latently infected and do not normally produce infectious KSHV unless they are stimulated by phorbol esters or other chemicals in cell culture. It is possible that a low level of KSHV-infected

BCBL-1 cells undergoes spontaneous lytic gene expression in vitro and in vivo, but this virus shedding a priori must not be required for tumor formation. Therefore, it was surprising to find that BCBL-1 tumors implanted in nude mice appeared to efficiently activate KSHV's lytic replication as evidenced by the expression of the lytic genes ORF59 and K8.1. Thus, it appears that some level of lytic replication may be important in tumor growth. Additional experiments are needed to clarify this issue.

In our study, we successfully developed a siRNA-induced conditionally silencing system. This system allowed us to investigate the role of the carboxyl terminal region of KSHV gB in virion egress and infectivity and the role of gB in tumorigenicity. However, transient transfection of siRNA in BCBL-1 and 293 cells in our previous studies provided only 10-15% transfection efficiency. Moreover, transient expression cannot be maintained in dividing progeny cells. Lentiviral RNAi mediated gene silencing system is one of the possible ways to promote transfection and transduction efficiency in vitro and in vivo. Therefore, the next logical step of this line of investigation is to produce gB-null BCBL-1 cells by permanent expression of anti-gB siRNAs. Establishment of these cells will be instrumental in dissecting the structure and function of KSHV gB in cell culture as well as in vivo animal model systems aimed at assessing potential functions of gB in virally-induced angiogenesis and tumorigenesis. Currently, we are working on the establishment of the lentiviral RNAi system in BCBL-1 cells (Appendix).

It is generally believed that tumor progression is highly dependent on angiogenesis. Vascular endothelial growth factor (VEGF) is currently thought to be one of the most important angiogenic factors involved in inducing the degradation of the vessel basement membrane, which in turn allows cells to invade the surrounding matrix. It was shown that KSHV-infected cells secrete VEGF and induce in vitro angiogenesis. KSHV gB facilitates virus infection and

transformation via its interaction with VEGFR-3 (Zhang, Groopman, and Wang, 2005; Zhang et al., 2005). KSHV infected B cells can attach to extracellular matrix proteins via gB and cellular integrin in the presence of Matrigel (Dyson et al., 2008). However, the pathways leading to the angiogenic factor secretion by PEL cells is not fully understood. A number of previous studies showed that different factors may be responsible for the VEGF induction and its angiogenic effect. vIL-6 was shown to promote stimulation of VEGF in vitro and in vivo (Aoki et al., 1999; Liu et al., 2001). Raf was indicated to regulate the expression of a variety of growth factors including VEGF (Hamden et al., 2005). KSHV K1 glycoprotein was also shown to up-regulate the expression by activating the VEGF promoter (Wang et al., 2004). Very recently, VEGF and b-FGF secreted by PEL cells were found in the ascetic fluid of NOD/SCID mice (Haddad et al., 2008). Interestingly, preliminary studies in our laboratory have shown decreased expression levels of VEGF molecules in BCBL-1 cells transfected with anti-gB siRNAs (Subramanian, personal communication). It would be important to determine the molecular basis of this phenomenon.

SUMMARY

KSHV glycoprotein B (gB) is one of the most conserved herpesvirus glycoproteins. It is an essential virion component for members of alpha- and beta- herpesvirus subfamilies. Envelope-associated KSHV gB is important for viral attachment to cell-surface and virion entry via binding of the gB RGD to integrins. Previous studies have shown that KSHV gB is essential for virion egress from 293 cells. KSHV virus lacking gB was unable to egress from 293 cells. In contrast, virion egress was detected in cells containing exogenously provided gB.

I was fortunate to assist Dr. Ramesh Subramanian in delineating the role of the cytoplasmic terminus of gB in virion egress and infectivity. This work showed that the carboxyl

terminus of gB is not important for either virion infectivity or egress from BCBL-1 cells (Subramanian et al., 2008). I was able to show that BCBL-1 cells inhibited by anti-gB siRNAs were unable to develop continuously growing tumors in nude mice. This is an important result that has to be further investigated in the immediate future. It is highly probable that gB is directly involved in angiogenesis, since preliminary results in the laboratory appear to indicate that gB-inhibition down-regulates VGEF secretion from BCBL-1 cells (Subramanian, personal communication). This result has to be confirmed in vitro and in vivo by following specific markers for angiogenesis.

The production of BCBL-1 cells permanently transduced with siRNA cassettes through the use of the lentiviral vectors that have been already constructed will greatly facilitate future studies along the lines discussed above. It is also possible that multiple KSHV genes can be simultaneously silenced to determine the combined effect of multiple glycoproteins and other viral genes that may be involved in tumorigenicity. In this regard, the siRNA system may be proven to be a highly versatile system for the conditional silencing of multiple genes.

Similar work with the BCBL-1 cells can be also performed to study Kaposi's sarcoma (KS). In this line of investigation KSHV infection of microvascular endothelial cells can be used in conjunction with nude or SCID mice to address the role of gB and other viral genes in Kaposi's sarcoma induction.

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APPENDIX

GENERATION OF STABLE KSHV GB-NULL CELL LINES

Kaposi's sarcoma-associated herpesvirus (KSHV) or the Human Herpesvirus-8 was first identified in 1994 (Chang et al., 1994). KSHV is a member of the gamma-2-herpesvirus family of Rhadinovirus (Neipel, Albrecht, and Fleckenstein, 1997; Russo et al., 1996). KSHV is etiologically associated with Kaposi's sarcoma, primary effusion or body cavity-based lymphoma and multicentric Castleman's disease (Antman and Chang, 2000; Ganem, 1998; Schulz, Sheldon, and Greensill, 2002). In 1996, the KSHV genome derived from the BC-1 cell line was fully sequenced (Moore et al., 1996).

KSHV encodes numerous glycoproteins during its lifecycle. Many of these viral glycoproteins are crucial for virus entry and cell-to-cell spread (Knipe and and Straus, 2001). Glycoprotein B (gB) is conserved among all herpesvirus and it is thought to directly function in virion attachment, the fusion of viral envelope with cellular membranes and virus-induced cell fusion during virus entry into permissive cells (Cai, Gu, and Person, 1988; Cranage et al., 1986; Pereira, 1994). Virus-induced cell fusion further allows for the spread of herpesvirus to neighboring cells (Baghian et al., 1993; Foster, Melancon, and Kousoulas, 2001). KSHV gB is a structural component of the virion and is thought to mediate virus attachment to cell integrins on cell surfaces via a gB RGD amino acid motif. One of the striking features of KSHV gB is that carboxyl terminal mutations can enhance virus-induced cell fusion (Baghian et al., 1993; Foster, Melancon, and Kousoulas, 2001; Pertel, 2002). Also, KSHV gB is involved in virion egress in 293T cells, since a gB-null virus was unable to egress from 293T cells, while complementation by exogenous gB restored virion egress (Krishnan et al., 2005). These observations suggest that KSHV gB is important for both virion egress and infectivity.

Mutagenesis of viral genomes has been extensively used to assess the structure and function of individual viral proteins. However, in the case of KSHV, recombinant viruses that carry deletions and other mutations are very difficult to construct largely due to difficulties associated with recovering substantial amounts of infectious virions after mutagenesis and transfection of viral DNA into susceptible cells. .

The RNA interference (RNAi) pathway is active in mammals and RNAi has emerged as a highly efficient and powerful tool for investigating mammalian gene function. Compared to knockout genetics, RNAi-based silencing is rapid, cost effective, and can be easily adapted to study homologous gene function in a wide variety of organisms. RNAi depends on the formation of double-strand RNA (dsRNA), which has its antisense strand complementary to the transcript of a targeted gene. The dsRNA silences the targeted gene by either inducing a sequence-specific degradation of complementary mRNA, or by inhibiting translation (Mittal, 2004). Two distinct steps are involved (Hutvagner and Zamore, 2002b; McManus and Sharp, 2002). First, the enzyme Dicer cleaves long dsRNA into short interfering RNA (siRNA) molecules of 21-23 bp in length. Second, a multicomponent RNA-induced silencing complex (RISC) uses siRNA to guide the sequence-specific cleavage of the RNA transcripts of the target gene. Gene silencing by this mechanism has been accomplished by transfection of chemically synthesized siRNA into host cells, by-passing the "dicing" step, to silence or knockdown gene expression (McManus and Sharp, 2002). Chemically synthesized 21-22 nucleotides (nt) siRNAs were shown to degrade mRNA via the RNAi mechanism evading the interferon response (Elbashir, Lendeckel, and Tuschl, 2001).

Effective gene silencing primarily depends on three factors: target sequence selection, optimal delivery of siRNA to cells and persistence of siRNA expression. Several recently

developed vector-based systems that contain stem-loop construct hairpin RNAs are able to induce intracellular generation of siRNA-like species (Brummelkamp, Bernards, and Agami, 2002; McCaffrey et al., 2002; Paddison, Caudy, and Hannon, 2002; Paul et al., 2002; Sui et al., 2002; Xia et al., 2002; Yu, DeRuiter, and Turner, 2002). Dicer cleavage of hairpin RNA can generate small dsRNAs that silence expression of target genes whose transcripts are complementary to one of the two strands of the hairpin RNA (Hutvagner and Zamore, 2002b). However, most vector-based hairpin expression systems can only transiently inhibit gene expression. Until recently, long-term knockdown of specific genes was achieved using retroviral vector constructs that express hairpin RNAs in cell cultures (Barton and Medzhitov, 2002; Brummelkamp, Bernards, and Agami, 2002; Devroe and Silver, 2002; Paddison and Hannon, 2002). The system for retrovirus delivery of RNAi-based gene silencing can also be adapted to use in lentiviral systems (Stewart et al., 2003b) .

Lentiviruses have the unique ability among retroviruses of being able to infect non-dividing cells and expand the usefulness of the RNAi-based gene silencing system (Gropp and Reubinoff, 2007; Harper and Gonzalez-Alegre, 2008; Klinghoffer et al., 2008; Ren et al., 2007; Sumimoto and Kawakami, 2007). As the particles are often pseudotyped with the envelope of the vesicular stomatitis virus (VSV), the vector can serve to introduce genes into a broad range of tissues and can be used *in vivo*. Furthermore, it has been demonstrated to be an excellent *in vivo* expression system achieving gene expression for several months without detectable pathology.

RNAi-mediated inhibition of KSHV gene expression has been previously utilized to ascertain the function of specific viral genes. siRNA-mediated silencing of KSHV K13 expression in PEL cell lines substantially inhibited NF-κB production and apoptosis (Godfrey et

al., 2005; Guasparri, Keller, and Cesarman, 2004). Recently, we utilized siRNA to conditionally knockdown the expression of KSHV glycoprotein B in 293T and BCBL-1 cells. KSHV gB was then rescued by codon-optimized gB DNA, which could not be inhibited by the siRNA due to its altered sequence. To achieve a stable and efficient siRNA-mediated knockdown of gB, we have used a lentivirus-based expression system. The overall goal of this line of experimentation is to develop a durable lentiviral–RNAi based system that can effectively and permanently inhibit KSHV gB expression in BCBL-1 cells at much higher levels that the ones achieved with transient expression of siRNAs. BCBL-1 cells incapable of expressing gB can then be used to delineate the functional domains of KSHV gB involved in virion egress, infectivity and tumorigenicity, by supplying in trans modified gBs that are not subject to siRNA inhibition.

MATERIALS AND METHODS

Cloning and Preparation of Lentiviral Vectors

The construction of recombinant lentivirus expressing anti-gB siRNAs was accomplished by using the plenti6/V5-D-TOPO cloning system (Invitrogen). The pLenti6/V5-D-TOPO vector is designed for use with the ViraPower Lentiviral Expression System (Invitrogen). siRNAs were designed targeting the 5' region of KSHV gB expression sequence using the AMBION's online siRNA target finder. The siRNA 18 was located from the nucleotide 403 after the ATG codon, while siRNA22 located starting at 484 bases from the ATG codon. Two siRNAs were generated as follows: siRNA 18 sense strand (5'-CACCCAAGTATGAACTCCCGAGATTCAAGAGAT CTCGGGAGTTCATACTTGTT-3') and anti-sense strand (5'-AACAAGTATGAACTCCCAG ATCTCTTGAATCTCGGGAGTTCATACTTG-3'); siRNA22 sense strand (5'-CACCGGTAA ATGTCAACGGGGTATTCAAGAGATACCCCGTTGACATTTACCTT-3') and anti-sense strand (5'-AAGGTAAATGTCAACGGGGTATCTCTTGAATACCCCGTTGACATTTACC-3').

The siRNAs contained the sequence CACC at the 5' ends of sense strands respectively for cloning purposes. The siRNA 18 and siRNA 22 were denatured at 94 ºC for 5 minutes and cooled down slowly to room temperature to allow annealing of the two strands. To produce lentiviral-siRNA expression clones, each annealed siRNA was TOPO-cloned into the pLenti6/V5-D-TOPO vector. After TOPO cloning, One Shot Stbl3 chemically competent *E. coli* cells (Invitrogen) were transformed with DNA according to the manufacturer's protocol and spread on LB agar plate containing 100 μg/ml ampicillin. All constructs containing siRNAs were verified by BamHI/XhoI restriction enzymes digestion and sequenced to confirm their structure.

Transfection

The 293FT lentiviral packaging cell line was obtained from the ViraPower Lentiviral Expression Kit (Invitrogen). 293FT cell lines were cultured according to the manufacturer's instructions. 293FT cells were cultured in a six-well plate in 2ml of antibiotic-free DMEM/10% heat inactivated fetal bovine serum (FBS) medium. Transfection was done until 293FT cells replicated to 80% confluence. 293FT cells were transfected with 1μg pLenti6/V5-D-TOPO vector and 3μg ViraPower packaging mix (Invitrogen) using 12μl LipofectAMINE 2000 (Invitrogen) in 1ml OptiMEM transfection medium without serum (Invitrogen). After overnight, media was removed and 3ml of antibiotic-free DMEM/10% FBS was supplied. The 293FT cells were allowed to produce lentivirus for 72h post-infection. Media containing live lentivirus was harvested and spun-down to remove cell debris before storage at -80 °C.

Infectivity of Lentiviruses in BCBL-1 Cells

BCBL-1 cells were allowed to grow to 60% confluence in T75 flasks. Cells were spun-down and suspended in 0.5ml cell culture media per well of 12-well plate. Cells were infected with

0.5ml lentivirus and polybrene (hexadimethrine bromide) at a final concentration of 8μg/ml. Cells were infected for 12h before replacement of another 1 ml cell culture media. BCBL-1 cells were incubated for 72h before the aspiration of cell culture media. To select stable cell line, blasticidin (Invitrogen) was used at a final concentration of 5-7.5μg/ml. Cells were cultured in the presence of blasticidin for 20 day to ensure stable cell line selection.

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VITA

Haixia Kong was born in January, 1982, to Youlan Wu and Ronggui Kong in Zhenjiang, China. Instantly, she and her three-year-old big sister became lifelong friends; her sister Haiyan Kong was born in 1979. In 2000, Haixia graduated with special veterinary-track honors from Dagang High School, and she participated in the Olympics of Chemistry. Haixia graduated from the College of Veterinary Medicine at Nanjing Agricultural Univeristy in 2004 earning many scholarships and honors. After graduation, Haixia was awarded a special honor to pursue advanced research in Preventive Veterinary Medicine at Zhejiang Unviersity for two years. In 2006, Haixia came to the United States and joined the laboratory of Dr. Kousoulas pursuing a Masters of Science (MS) degree working on the molecular biology of Kaposi's sarcoma-associated herpesvirus glycoproteins. After graduation, Haixia will pursue her veterinary license to practice veterinary medicine in the USA. She hopes to broaden her understanding of veterinary medicine and molecular biology in an attempt to improve herself, her discipline and her community.