Epstein-Barr Virus Inhibits Kaposi’s Sarcoma-Associated Herpesvirus Lytic Replication In Primary Effusion Lymphomas

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Running title: Interaction between EBV and KSHV in PEL

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Abstract

The majority of AIDS-associated primary effusion lymphomas (PEL) are latently infected with both Kaposi sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). PELs harboring two viruses have higher oncogenic potential, suggesting functional interactions between EBV and KSHV. KSHV replication and transcription activator (K-RTA) is necessary and sufficient for induction of KSHV lytic replication. EBV latent membrane protein 1 (LMP-1) is essential for EBV transformation and establishment of latency in vitro. We show EBV inhibits chemically-induced KSHV lytic replication, in part because of a regulatory loop in which K-RTA induces EBV LMP-1, and LMP-1, in turn, inhibits K-RTA expression, and furthermore the lytic gene expression of KSHV. Suppression of LMP-1 expression in dually infected PEL cells enhances the expression of K-RTA and lytic replication of KSHV upon chemical induction. Because LMP-1 is known to inhibit EBV lytic replication, KSHV mediated induction of LMP-1 would potentiate EBV latency. Moreover, KSHV infection of EBV latency cells induces LMP-1, and K-RTA is involved in the induction. Both LMP-1 and K-RTA are expressed during primary infection of EBV to KSHV latency cells. Our findings provide evidence that an interaction between EBV and KSHV at molecular levels promotes the maintenance and possibly establishment of viral latency, which may contribute to pathogenesis of PELs.
Introduction

Both Epstein-Barr virus (EBV) and Kaposi sarcoma (KS)-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV-8), are members of human gamma herpesviruses, and associated with a variety of human malignancies. EBV is an important cause of lymphomas in severely immunocompromised persons, especially patients with AIDS and organ-transplant recipients (46, 61, 62, 64, 66). KSHV is believed to be the etiological agent of KS (16, 18, 60), and is implicated in the pathogenesis of AIDS-associated primary effusion lymphoma (PEL), also called body cavity-based lymphoma (BCBL), and multicentric Castleman’s disease (23, 60, 86).

Like other herpesviruses, EBV and KSHV go through both latency and lytic replication cycles. KSHV replication and transcription activator (K-RTA) is necessary and sufficient for the switch from latency to lytic replication in KSHV (23, 86, 88). K-RTA is a sequence-specific DNA-binding protein that regulates many subsequently expressed viral genes (38, 56, 69, 70, 75, 91, 97). Also, K-RTA can interact with other factors to modulate its transcription potential (39, 40, 51-53).

EBV latent membrane protein-1 (LMP-1) is required for EBV transformation of primary B cells and establishment of EBV latency in vitro and believed to play similar roles in EBV-associated tumor cells in vivo (45, 47). LMP-1 is an integral membrane protein and acts as a constitutively active receptor-like molecule that does not need a ligand (30, 36, 46, 54). LMP-1 activates a variety of cellular genes that enhance cell survival, adhesive, invasive, angiogenic, and antiviral potential (31, 41, 59, 81-83, 87, 90, 94, 95).

Interestingly, the majority of PELs are harboring with both EBV and KSHV (13, 14, 42, 68). In order to understand their contributions to the pathogenesis of PEL, it is important to address how EBV and KSHV interact with each other and affect biological properties of the cell and the viruses. PELs harboring two viruses have higher oncogenic potential (76), suggesting potential interactions between EBV and KSHV. This report describes a molecular interaction between EBV and KSHV, i.e., KSHV
K-RTA potentiates EBV latency via induction of EBV LMP-1 and uses LMP-1 to curb KSHV lytic replication. These data suggest that the coinfection of EBV and KSHV in the majority of BCBLs might not be a coincidence: the presence of EBV is one of the strategies that KSHV uses for the maintenance of its latency. This report should be applicable to both KSHV and EBV studies in the majority of AIDS-associated PELs.

Materials and Methods

Cell Culture, Plasmids, Adenoviruses, and antibodies. All cell lines used in this study and their properties are listed in Table 1. Expression plasmids of K-RTA (pCMV50), the recombinant adenovirus for green fluorescence protein (GFP) (AdGFP) and K-RTA (AdRTA) were a gift from Dr. Byrd Quinlivan (71). K-RTA K152E mutant is a DNA-binding mutant of K-RTA (91). The recombinant adenovirus for LMP-1 (AdLMP1) was gift from Drs. Stephen Gottschalk and Cliona Rooney (37). The β-galactosidase expression plasmid, pCMVβ, was purchased from Clontech. LMP-1 and LMP-DM, which is a mutant LMP-1 failing to initiate signals, are described (94). The LMP-1 promoter reporter constructs were generated by inserting PCR products into the pGL3-basic vector (Promega). LMP-mISRE luc is exactly the same as LMP-ISRE-luc except the ISRE mutation. The LMP-1 ISRE (5'-AGGAAATGGAAAGG-3') was mutated at the underlined nucleotides for mISRE (5'-AGGGGGTGGGGG-3'). The pHp vector was constructed by ligation of the PacI/blunt-XbaI fragment of pAdTrack-HP (96) into the Asel/blunt-XbaI sites of pEGFP-N1 (ClonTech). The sequences, 5'-TGGAACGCGACCTTGAGAG-3' was used to clone the small interfering RNA for LMP-1 (siLMP-1) expression plasmid in PHP. 5'-TGGCGCAAGATGACAAGG-3' was used for making siRNA for K-RTA (siKRTA). The sequence for p53 was described (96). All constructs were verified by sequence analysis by the UNL Sequencing Facility. K-RTA antibody was from Dr. David Lukac (All figures except Figure 1). Rabbit polyclonal antibody against K-RTA peptide (amino acids 667 to 691), “ATPANEVQESGTLYQLHQWRNYFRD”, was customer made by Alpha Diagnostic.
International. This K-RTA antibody was used for Figure 1. K8 antibody was from Dr. Jae Jung. Tubulin antibody was purchased from Sigma. EBV E-RTA and EA-D antibodies were purchased from Argene, LMP-1 antibody (CS1-4) was from Dako, STAT-1 monoclonal antibody was from Santa Cruz. Rat monoclonal antibody to KSHV LANA was obtained from ABI. Secondary goat-anti-rat conjugated with cy5 was from Jackson ImmunoResearch.

Enrichment of transfected cells and making cell lines Enrichment for CD4 positive cells was performed with the use of anti-CD4-antibody conjugated to magnetic beads according to the manufacturer’s recommendation (Dynal). The isolated cells were lysed immediately and used for western blot analysis. However for chemical treatment, cells were detached from the Dynabeads CD4 by incubation for 45-60 minutes at room temperature with 10 µl of DETACHaBEAD (Dynal). The detached beads were removed by using a magnet separation device. The released cells were washed 2-3 times with 500 µl RPMI 1640 plus 10% FBS, and resuspended in RPMI 1640 plus 10% FBS at 5x10^5 cells/ml. Cells were recovered for 2-6 hours before the addition of chemical reagents. BC1 (EBV+/KSHV+) cells were transfected with either PHP (control) or PHP-siLMP-1 (siLMP-1) along with CD4 expression plasmid, and the transfected cells were enriched and cultured in the presence of G418 (1 mg/ml) and 20% FBS for one week for making cell lines. The cells were then treated by chemicals for induction of lytic replication.

Preparation of KSHV stocks and in vitro infections BCBL-1 cells were treated with TPA (30 ng/ml) and sodium butyrate (3 mM) to induce the lytic cycle of KSHV replication, and culture supernatants were harvested 7 days later. Virions were pelleted by centrifugation at 100,000 xg for 1 hour. Virion pellets were subsequently resuspended in complete medium (1:100 of the volume of the original supernatants) and were passed through a 0.45-µm filter. For infection, 293-EBV or BZLF1-KO cells at approximately 50% confluence were incubated with concentrated viruses. After
incubation at 37°C for 24 hours, cells were washed and cell lysates were used for detection of target proteins.

**Preparation of EBV stocks and in vitro infections**  EBV lytic replication of AGS-BX1-g cells was induced by 0.5 mM sodium butyrate and 30ng/ml of TPA. Media will be replaced with fresh ones without TPA and butyrate after 24 hrs. Media were collected four days later with or without concentration and passed through 0.45-µm filter. For infection, Cro6 cells were incubated with 0.5 or 1 ml of EBV stock. After incubation at 37°C for 24 hours, cells were collected and RNA was isolated for RT-PCR analysis. Infectivity was examined by detecting GFP-positive cells under UV microscope. Similar results were obtained with concentrated or non-concentrated EBV viruses (Data not shown).

**Transient Transfection, adenovirus infection and Reporter Assays**  Effectene (Qiagen) was used for the transfection of 293EBV, BRLF1-KO and 293T cells. Electroporation were used for transfection of BC3 and DG75 cells. The recombinant adenoviruses were titered in 293 cells. BRLF1-KO cells were infected with adenovirus at a multiplicity of infection (MOI) of 5 (calculated from PFU), while MOI of 100 was used for infection of BC3 cells. The luciferase assays were performed using the assay kit from Promega according to manufacturer’s recommendation.

**Electrophoretic mobility shift assay (EMSA)**  Expression and purification of KSHV RTA proteins have been described (91). The probes were obtained by first annealing complementary oligonucleotides and then labeling them with [α-32P]dCTP (Amersham) using DNA polymerase Klenow Fragment (Fermentas). LMP-1 ISRE probe was obtained by annealing two oligonucleotides, 5’-GATCGCAACAGGAAATGGAAAGGC-3’ and its complementary stand, with GATC at the 5’ end. EMSA was performed essentially as described (73, 91).

**Induction of KSHV lytic replication.**  BC3 were treated with 5-10 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA) for 18-24 hrs to induce lytic viral replication. For BC1 cells, 0.5-1 mM sodium
butyrate was used for 18-24 for the induction of KSHV lytic replication. For experiments in Figure 1, BC3, BC3EBV (cl10), Cro6, Cro6EBV(cl2) were treated with sodium butyrate at 0.5-1 mM.

**Western Blot Analysis, RNA extraction, RNase Protection Assays (RPA), and RT-PCR.**

Standard western blot analysis was performed as described (92). Total RNA was isolated from cells using the RNeasy Total RNA Isolation Kit (Qiagen). RPA was performed with total RNA using the RNase Protection Assay Kit II (Ambion) as described (93). The GAPDH probe was from US Biochemicals, Inc. The probe for LMP-1 was a gift from Dr. Joseph Pagano. Reverse transcription was performed with the use of the SuperScript reverse transcription system from Invitrogen following the manufacturer’s recommended protocol. Reverse transcribed cDNA (2µl) was then PCR amplified with the appropriate primers. PCR conditions were as follows: initial denaturation at 95°C for 3 min followed by 30-35 cycles of 95°C for 30 seconds, 62°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified DNA was then resolved on 8% PAGE. Primers used for LMP-1 were: 5’-TGATCATCATTTATCTTCAAGACCTT-3’ and 5’-CCTGTCCGTGCAAATTCCA-3’. For K-RTA: 5’-CGCAATGCGTTACGTTGTTG-3’ and 5’-GCCCGGACTGTTGAATCG-3’. For actin: 5’-TTCTACAATGAGCTGCGTGT-3’ and 5’-GCCAGACAGCACTGTGTGGG-3’

**Cell lysate and immunoprecipitation** Cells (10⁸) were collected by centrifugation and were resuspended in 1 ml of extraction buffer (0.5% NP-40, 0.3M NaCl, 0.1mM EDTA, 20mM Hapes (7.4), 1mM DTT, and 10% glycerol), supplemented with complete protease inhibitors (Roche). The cell suspension was kept in 4 °C for 30 minutes with gentle agitation. Insoluble material was pelleted at 14,000 x g for 15 min at 4 °C, and the supernatant was used directly for immunoprecipitation. A specific antibody was added to the lysates, followed by addition of 40 µl of protein G Sepharose (Pharmacia), and the sample was incubated for overnight at 4 °C with continuous mixing. Protein immunocomplexes were isolated by centrifugation, and beads were washed three times with extraction buffer.
buffer. Immunoprecipitated proteins were resuspended in SDS-PAGE loading buffer, and analyzed by SDS-PAGE and western blot analyses were employed with specific antibodies.

RESULTS

EBV inhibits the lytic replication of KSHV Because the majority of the current studies on KSHV and EBV use single-infected cells as a model system, the potential effects of EBV and KSHV on each others’ biology obviously need attention. We thus examined the effect of EBV on the KSHV lytic replication process. BC3 and Cro6 are KSHV+/EBV- PEL lines. Isogenic dually infected cell lines were generated by EBV infection (76). The expression of the entire panel of EBV latent genes, including EBV nuclear antigen 1(EBNA1), 2, 3, 4, 6, LMP1, 2A, 2B, and EBV-encoded small RNAs (EBERs), was examined in these isogenic cell lines. All EBV-infected clones of the PEL cells expressed EBNA-1, EBER-1, and LMP2A. EBNA2-6 was not expressed in any of the converted lines. The expression of LMP1 was heterogeneous as determined by western blot analyses: Cro6-EBV (cl 2) expressed LMP-1 protein. BC3-EBV (cl 10) had no detectable LMP-1 protein levels (76). We now show that LMP-1 is expressed in both BC3-EBV and Cro6-EBV lines in RT-PCR assay (Supplementary Figure 1). All these data suggested that EBV formed type II latency with low levels of LMP-1 expression in these EBV convertants used in present study, which is in agreement with previous reports about EBV status in dually infected cells (11, 26, 42, 50).

Most isogenic cell lines are made and cultured for some time, and have not encountered any major loss of EBV primarily due to the fact that these EBV infected PELs are maintained in G418 selection. Because the recombinant EBV containing GFP was used for the establishment of isogenic lines, expression of GFP can be used to identify these EBV-positive cells. We found that these EBV-convertants are 100% positive for KSHV as determined by the expression of KSHV latency-associated nuclear antigen (LANA, also called ORF73) (Supplementary Figure 2A), and 65% and 85% GFP
positive for BC3EBV and Cro6EBV respectively (Supplementary Figure 2B). Thus, BC3EBV and Cro6EBV have 65 and 85% cells respectively that are dually infected by both KSHV and EBV.

KSHV lytic replication can be induced by some chemicals, such as sodium butyrate. The cells were treated with same amounts of sodium butyrate, and the expression of KSHV lytic gene expression was examined by western blot analyses. As shown in Fig. 1, the expression of K-RTA and K8 genes were detected after the treatment; indicating the initiation of lytic replication processes. However in the presence of EBV, K-RTA and K8 were expressed at lower levels (Fig. 1). Because both K-RTA and K8 are essential genes required for KSHV lytic replication (6, 43, 88), these results suggested that EBV inhibited sodium butyrate-induced KSHV lytic replication.

**KSHV RTA induces the expression of EBV LMP-1 protein** EBV LMP-1 promoter region has three kinds of cis elements through which K-RTA could possibly regulate: RBP-Jκ binding sequence (RBP-Jκ), interferon-stimulated response element (ISRE), and gamma interferon activation sequence (GAS) (40, 51, 52, 91) (Fig. 4A). We thus tested if K-RTA could induce the expression of LMP-1. The recombinant adenoviruses containing K-RTA (AdRTA) or GFP (AdGFP) were used to infect a BRLF1-KO cell line (EBV+/KSHV-) (27). EBV BRLF1 gene product (or E-RTA) has been shown to induce the expression of LMP-1 (17). The use of the BRLF1-KO cells would eliminate the potential contribution of EBV BRLF1 to LMP-1 induction. As shown in Fig. 2A, endogenous LMP-1 expression was increased significantly in response to AdRTA, but not to AdGFP infection.

BRLF1-KO cells are generated in human 293 cells in which recombinant adenovirus can replicate. In order to eliminate the possible contribution from adenovirus replication, we examined the effect of K-RTA in transient transfection assays. Expression plasmid of K-RTA or EBV homologue of K-RTA, E-RTA (EBV BRLF1 gene product) was transfected into BRLF1-KO cells. As shown in Fig. 2B, K-RTA could induce the expression of LMP-1; however, E-RTA could not induce the expression of LMP-1 to similar levels as K-RTA did. The inability of E-RTA to induce LMP-1 might
be due to the differences in cellular background (17). Because LMP-1 is expressed during EBV lytic replication cycle (8, 20, 67), it is thus possible that K-RTA might induce EBV lytic replication that results in the synthesis of LMP-1. However, this scenario is unlikely because it is well documented that K-RTA is unable to induce the lytic replication of EBV and we confirmed the observation [(74), and data not shown]. The results thus suggested that K-RTA-mediated LMP-1 induction is not related to activation of EBV lytic replication.

**Induction of LMP-1 protein by K-RTA is independent of EBNA-2**  
Because EBV nuclear antigen 2 (EBNA-2) is the primary activator of EBV LMP-1 (1, 35, 77, 84), it is possible that K-RTA induces the expression of EBNA-2 that in turn activates LMP-1. P3HR1(EBV+/KSHV-) is a Burkitt’s lymphoma (BL) line that harbors an EBV genome without EBNA-2 (2). K-RTA expression plasmids were transfected into P3HR-1 cells along with a CD4-expression plasmid, and the levels of LMP-1 were determined after selection of the transfected cells by the use of CD4 antibody-conjugated magnetic beads (see Materials and Methods for detail). As shown in Fig.2C, K-RTA induced a marked increase in LMP-1 protein levels in P3HR1 cells. Because of the deletion of EBNA-2 in P3HR1 EBV genome, these results indicated that the induction of LMP-1 by K-RTA is, at least partially, independent of EBNA-2. The mutant, K-RTA-K152E, was unable to induce LMP-1. K-RTA-K152E also failed to induce LMP-1 protein in BRLF1-KO cells (data not shown). Because K-RTA-K152E has defect in DNA binding capability, DNA binding activity of K-RTA might be an important component of the induction of LMP-1 (91). K-RTA also induced the expression of LMP-1 in EBV latency cells with both EBNA2 and BRLF1 in the viral genome; suggesting the induction was not a cell specific phenomenon.

**K-RTA induces EBV LMP-1 at the RNA level** Next, we examined whether K-RTA induces LMP-1 at the RNA level. The K-RTA or its mutant K-RTA-K152E was transfected into BRLF1-KO cells. Total RNA was isolated from transfected cells 24 hours later. RNase protection assays (RPA) were
used for detection of LMP-1 RNA. K-RTA-transfected cells have higher LMP-1 RNA levels than that of vector or K-RTA-K152E transfected cells (Figs. 3A; 3B). The expression levels of K-RTA and its mutant were similar (Fig. 3C). Thus, K-RTA-mediated induction of LMP1 occurs at the RNA level.

**K-RTA activates LMP-1 promoter** There are at least three potential cis-elements that may be used by RTA for transactivation: RBP-Jκ, ISRE, and GAS (40, 51, 52, 91). K-RTA and various LMP-1 promoter reporter constructs (Fig. 4A) were transfected into 293T cells and the promoter activities were measured. The expression levels of K-RTA and its mutant were similar (Supplementary Figure 3A). As shown in Fig. 4B, the K-RTA was able to activate empty pGL3basic 2-6-fold under our conditions (columns 1-2), which is a background issue. The LMP-ISRE-luc reporter was strongly activated by K-RTA (columns 3-4), but the point mutations or deletion of ISRE crippled activation by K-RTA (columns 6-9). In agreement with the data in Figs. 2C, 3A, K-RTA-K152E mutant failed to activate LMP-1 promoter reporter constructs (column 5). Because LMP-ISRE-luc contains ISRE, RBP-Jκ, and GAS (Fig. 4A), these data suggest that the LMP-1 ISRE is a necessary cis element in response to K-RTA in the LMP-1 promoter.

Whether the LMP-1 promoter constructs are responsive to physiological levels of K-RTA was examined. In BC3 (KSHV+/EBV-) cells, sodium butyrate induced 10-fold activation of LMP-ISRE-luc, however, only 2-3 fold activations were observed when pGL3-basic or LMP-mISRE-luc were used for transfection (Fig. 4C). Moreover in DG75 (EBV-/KSHV-) cells, sodium butyrate could not activate LMP-ISRE-luc reporter construct (data not shown). These data suggest that a viral gene(s) in the lytic replication cycle may be responsible for activation of the LMP-1 promoter via LMP-1 ISRE. K-RTA is among the earliest gene products expressed in lytic replication and its expression was confirmed by Western blot analyses (Supplementary Figure 3B). Thus, the physiological levels of K-RTA might also activate the LMP-1 promoter construct.
K-RTA binds to LMP-1 promoter Finally, because K-RTA is known to bind to ISRE (91), it is expected that K-RTA could bind to the LMP-1 ISRE. We first tested if LMP-1 ISRE was able bind to endogenous K-RTA in lysates from PEL cells with lytic replication. However, we did not detect any specific binding (data not shown). Partially purified K-RTA protein, which has been used widely to study K-RTA binding (22, 55, 72, 73, 85), was then used for EMSA with LMP-1 ISRE probe. The purification process was following our previous report and partially purified K-RTA has the expected properties (91). Same amounts of partially purified proteins were used for EMSA. The binding of K-RTA, but not K-RTA K152E, to LMP1 ISRE probe was observed (Fig. 4D). Some bands were regularly appeared with our purified K-RTAs. The purified BSA was used to detect these non-specific binding patterns. In addition, cold LMP-ISRE was able to compete for the binding to K-RTA, and the K-RTA-DNA complex could be supershifted by the use of K-RTA-specific antibody (Supplementary Figure 3C). The purified proteins have similar purities as determined by Coomassie blue staining (Supplementary Figure 3D). These data suggested that K-RTA was able to bind to LMP-1 ISRE specifically in vitro as expected.

LMP-1 negatively regulates the lytic replication of KSHV EBV LMP-1 has been shown to suppress EBV reactivation triggered by either anti-IgM or TPA (3, 63). In addition, we have shown that LMP-1 is an antiviral protein (87, 90). These published results suggest that LMP-1 might inhibit the lytic replication of KSHV.

BC-3 cells (KSHV+/EBV-) were transfected with LMP-1 or LMP-DM, a LMP-1 mutant that fails to induce an antiviral state and to inhibit the lytic replication of EBV (63, 90). The transfected cells were enriched and recovered for several hours before the addition of TPA (see Materials and Methods for detail). As shown in Fig. 5A, LMP-1 significantly reduced the expression of K-RTA and K8. Expression of another KSHV lytic gene, vIRF1, was also inhibited (data not shown). Furthermore, LMP-DM, which fails to initiate signals from LMP-1 (94), failed to inhibit KSHV lytic
replication; suggesting that the signals derived from LMP-1 are involved in the inhibition. The recombinant adenovirus containing LMP-1 was also used to infect BC3 cells. As shown in Fig. 5B, while the infection by AdGFP still allowed significant lytic replication, the infection of AdLMP-1 inhibited the expression of K-RTA as well as K8. Thus, overexpression of EBV LMP-1 inhibits KSHV lytic replication.

**Suppression of LMP-1 enhances KSHV lytic replication** Having established that overexpression of LMP-1 inhibits KSHV lytic replication, we then examined if endogenous LMP-1 was involved in the lytic replication of KSHV in dually-infected PEL cell lines. A small interfering RNA for LMP-1 (siLMP-1) expression plasmid has been generated and was able to suppress the expression of LMP-1 significantly in transient transfection assays in 293T cells (Fig.6A). The siLMP-1 was used to generate a stable cell line in BC1 cells, which is a PEL line dually infected with both EBV and KSHV, and was able to suppress endogenous LMP-1 expression (Fig. 6B). Sodium butyrate was able to selectively induce the lytic replication of KSHV but not EBV (74). Upon the induction of KSHV lytic replication by sodium butyrate, the siLMP-1 expressing cells have higher expression of K-RTA and K8 than that of control cells (Fig. 6C). LMP-1 protein was detectable in several dually-infected PEL lines by immunoprecipitation (IP)-western blot analysis (Fig. 6D). The majority of LMP-1 proteins were lost during the immunoprecipitation process under our experimental condition; the use of IP to show the quantitative differences was unreliable. In addition, immunostaining of LMP-1 and K-RTA in dually infected BC1 cells after treatment of sodium butyrate showed that the majority of cells were either K-RTA or LMP-1-positive, and only a small fraction (<10%) were positive for both (data not shown). All these data suggested that endogenous LMP-1 was contributing to the control of KSHV lytic replication in dually-infected PELs.

**Expression of LMP-1 during primary infections** To address if the interaction between K-RTA and LMP-1 is relevant to the establishment of latencies in dually-infected PELs, we examined if LMP-1 is
induced during primary infections. First, whether LMP-1 is inducible during KSHV infection of EBV latency cells was examined. 293-EBV cells (KSHV-/EBV+) were used for the infection by concentrated KSHV. The reason to choose 293EBV is that KSHV apparently infects fibroblasts efficiently (49). Given the fact that infection of KSHV to B cells is inefficient (19, 44), the infection of 293EBV cells may be the best available alternative to address alterations in EBV gene expression during primary infection of KSHV. As shown in Fig. 7A, LMP-1 is highly induced by KSHV infection. K-RTA is expressed during primary infection (49). Whether K-RTA is involved in the induction of EBV LMP-1 in primary infection was addressed by the use of siRNA for K-RTA. After the transfection of siRNA for K-RTA into 293-EBV cells, the expression of both LMP-1 and K-RTA was reduced upon KSHV infection; suggesting the involvement of K-RTA in the induction (Fig. 7B).

Second, we examined if LMP-1 is expressed during EBV infection of KSHV latency cells. EBV was used to infect Cro6 (KSHV+/EBV-) cells. RNA was isolated from infected cells for RT-PCR analyses 24 hours later. The infection of KSHV latency cells by EBV was associated with EBV LMP-1 expression (Fig. 7D). Therefore in both scenarios, LMP-1 is expressed during primary infections.

**Induction of lytic replications during primary infection** EBV latency can be disrupted by several treatments including viral superinfection (4, 21, 29). To test what may happen in EBV latency, we examined if KSHV could disrupt EBV latency by inducing lytic replication of EBV. EBV EA-D (BMRF-1) expression was used as a marker for lytic replication. The essential function of EA-D in EBV lytic replication has been well established and using EA-D as an indicator of lytic replication has been appreciated in the field for years (28, 32, 34, 78-80, 89, 90). KSHV infection of 293EBV induced EBV EA-D expression (Fig. 7C). Thus, KSHV infection of EBV latency cells induced EBV lytic replication. However, KSHV infection still induced LMP-1 in BZLF1-KO (KSHV-/EBV+) cells, in which EBV lytic replication could not be induced (27) (Fig. 7C); suggesting that EBV lytic replication plays a limited role in the induction of LMP-1 by KSHV.
Finally, EBV was used to infect KSHV+/EBV- PEL cells. Because the infection of EBV to Cro6 cells is inefficient (<5%, data not shown), RT-PCR was used to detect the expression of K-RTA expression. As shown in Fig. 7D, infection of Cro6 (KSHV+/EBV-) cells by EBV induced K-RTA expression. Due to well-established functions of K-RTA, the results also suggested that EBV infection might induce lytic replication of KSHV.

**DISCUSSION**

By using isogenic cell lines, we discovered that EBV inhibited the lytic replication of KSHV (Fig. 1). Support for this conclusion also comes from the finding that TPA induces KSHV lytic replication in KSHV single-infected, but not in dually infected PELs (58), and an apparent negative correlation between spontaneous KSHV lytic gene expression and EBV infection in primary PEL specimens (25). It is also possible that KSHV inhibits EBV reactivation because sodium butyrate and/or TPA induce EBV lytic replication in EBV-single infected cells, but in dually-infected BC1 cells, only TPA induces EBV lytic replication (58). Because we were unable to generate dually infected lines in EBV latency cells, the direct effect of KSHV on EBV lytic replication could not be tested (data not shown).

To address the potential mechanism of EBV-mediated inhibition of KSHV lytic replication, we found that K-RTA in EBV latency cells induces LMP-1 in an EBNA-2-independent manner at RNA level (Fig. 2, 3). K-RTA activates LMP-1 promoter reporter constructs, and binds to LMP-1 promoter region (Fig.4). Overexpression of LMP-1 in PEL cell lines inhibited chemical-mediated induction of lytic replication processes (Fig. 5). The suppression of LMP-1 expression in dually infected PEL cells enhanced the lytic replication process (Fig. 6). Therefore, a regulatory loop between two human herpesviruses (KSHV and EBV) is identified, i.e., K-RTA induces LMP-1; and LMP-1 in turn down-regulates K-RTA.
Based on the fact that dually infected cells do express LMP-1 (Fig. 7D), it is likely that the endogenous LMP-1 plays a role in the control of KSHV lytic replication and the regulatory loop between K-RTA and LMP-1 may be responsible for the observed inhibitory effects of EBV on KSHV lytic replication (Fig. 1). In addition, LMP-1 is known to inhibit EBV lytic replication (3, 63). Thus, KSHV would potentiate EBV latency by inducing LMP-1. Due to the well-established functions of LMP-1 on cellular transformation and invasive potential, the regulatory loop between K-RTA and LMP-1 may also contribute to the pathogenesis of AIDS–associated PELs.

Because K-RTA is necessary and sufficient for the KSHV lytic replication, LMP-1-mediated inhibition of KSHV lytic replication is at least partially via the inhibition of K-RTA expression. EBV LMP-1 has at least two mechanisms for the inhibition of EBV lytic replication, and one of them is to downregulate the expression of essential genes for EBV lytic replication such as E-RTA (3). NF-κB has been shown to be a negative regulator of gamma herpesviruses lytic replication (10). LMP-1, but not LMP-DM, induces NF-κB activity (94). Because LMP-DM failed to inhibit KSHV lytic replication, LMP-1-mediated NF-κB activation is apparently associated with the inhibition of KSHV lytic replication. Thus, the finding that LMP-1 inhibits lytic replication process of KSHV might be in agreement with previously identified mechanisms for LMP-1 to control EBV lytic replication.

How both viruses establish their latencies in PEL cells is unknown. It is known that viral infection is a common inducer of EBV lytic replication (90). As expected, KSHV infection of EBV latency cells induces EBV lytic replication (Fig. 7C). In addition, EBV infection of KSHV latency cells might trigger the KSHV lytic replication by induction of K-RTA (Fig. 7D). It is very interesting to address how the two viruses could establish latencies in the same cells. LMP-1 is expressed either during KSHV infection of EBV latency cells or EBV infection of KSHV latency cells (Fig. 7). Therefore, the antiviral and repression of reactivation properties of LMP-1 plus its temporal expression during the primary infections may inhibit both EBV and KSHV lytic replications and
promote latency establishment of both viruses during primary infections. Of note is that LMP-1 protein may be present in EBV virion, and is possibly delivered into cells directly during the infection (24, 57), which could have a profound effect on the outcome of infection.

In summary, this report shows that two human oncogenic herpesviruses interact at the molecular level to reinforce each other’s latency in the same tumor cells, and the regulatory loop of K-RTA and LMP-1 may contribute to dual latency and the pathogenesis of PELs.

Acknowledgements

We thank Drs. H.J. Delucuse, David Lukac, Shannon Kenney, Byrd Quinlivan, Joseph Pagano, Jae Jung, Lindsey Hutt-Fletcher, Stephen Gottschalk, and Cliona Rooney for providing various reagents for this work, Dr. Yuan Yan for helpful discussion, and UNL Microscopy and Sequencing Facility for their assistance. Supported in part by grants from USDA 2005-01554 and 2006-01627 (CJ), and NIH R21AI59132, RO1CA108951, P20RR15635, and U54AI057160 (LZhang). TC was supported by NIH Ruth L. Kirschstein fellowship (T32 AIO60547). PT was supported by grants from MIUR, Associazione Italiana per la ricerca sul Cancro (AIRC) and from Ministero della Sanità, progetto AIDS. CK was supported in part by the Undergraduate Creative Activities & Research Experiences grant.

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binding by Kaposi's sarcoma-associated herpesvirus lytic switch protein is necessary for

product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for


**FIGURE LEGENDS**

**Table 1: Cell lines used in this study and their properties.** All the listed lines are human cell lines. The phenotypes, genotype, properties, and culture conditions are as shown.

**Figure 1. EBV inhibits KSHV lytic gene expression.**  
A. EBV inhibits the KSHV lytic gene expression in BC3 cells. BC3 and its EBV-infected counterpart, BC3-EBV, were treated with sodium butyrate (0.5 and 1 mM) for 12-16 hours. Lysates were separated on 10% SDS-PAGE, transferred, and used for western blot analysis. The membrane was cut in the middle and probed with different antibodies. The identity of proteins is as shown.  
B. EBV inhibits the KSHV lytic gene expression in Cro6 cells. Cro6 and its EBV-infected counterpart, Cro6-EBV, were treated with sodium butyrate for 12-16 hours. Lysates were used for western blot analysis. The same membrane was stripped and reprobed with other antibodies. One representative from three experiments is shown. The identity of proteins is as shown.

**Fig. 2. KSHV K-RTA induces the expression of EBV LMP-1.**  
A. recombinant adenovirus containing K-RTA induces the expression of LMP-1. Lysates from uninfected, AdRTA, or AdGFP infected BRLF1-KO cells were used for western blot analysis. BRLF1-KO contains EBV BRLF1-null genome. The same membrane was stripped and reprobed with other antibodies. The identity of proteins is as shown.  
B. Transfection of K-RTA induces the expression of EBV LMP-1. Lysates
from pcDNA3 (vector), K-RTA, or E-RTA transfected BRLF1-KO cells were used for western blot analysis. C. The induction of LMP-1 by K-RTA is independent of EBNA-2. K-RTA or K-RTA K152E plasmids were transfected into P3HR1 cells with CD4 expression plasmid, and the transfected cells were selected by CD4 magnetic beads. P3HR1 is an EBV+ line with EBNA2 deletion in the genome. Lysates from enriched cells were used for western blot analysis. The same membrane was stripped and reprobed with other antibodies. The identity of proteins is as shown.

**Fig. 3. K-RTA induces the expression of LMP-1 RNA.** A. K-RTA induces LMP-1 RNA. LMP-1 and GAPDH probes were labeled with $\alpha^{-32}P$-UTP and used for RPA. Yeast RNA, RNA from IB4 cells (positive control), or RNAs from the BRLF1-KO cells transfected with pcDNA3, K-RTA and K-RTA-K152E respectively were used for RPA analyses. Specific protections of LMP-1 and GAPDH RNAs are indicated. B. Relative LMP-1 RNA levels. Data were obtained by normalizing LMP-1 to GAPDH levels from three independent experiments with the use of a Gene Genius Bioimaging System. Standard deviations are shown. C. Expression levels of K-RTAs. Lysates from transfected cells were used for western blot analysis. The same membrane was stripped and reprobed with other antibodies. The identity of proteins is as shown.

**Fig. 4. KSHV K-RTA activates EBV LMP-1 promoters.** A. Schematic diagram of EBV LMP-1 promoter reporter constructs. RNA start site is shown. The drawing is not to scale. B. K-RTA activates EBV LMP-1 promoter reporter constructs. 293T cells were transfected with various reporter constructs along with CMV-$\beta$-gal, K-RTA or K-RTA-K152E expression plasmids as shown on the top. Luciferase activity was normalized by $\beta$-galactosidase activity. The folds of activation of promoter constructs are shown with standard deviations. One representative of 5 independent experiments is shown. C. LMP-1 promoter reporter is activated during KSHV lytic replication. BC3 is a KSHV+/EBV- PEL cell line. The reporter constructs were transfected into BC3 cells by electroporation. After several hours of recovery, the cells were treated with (+) or without (-) sodium
butyrate (3mM) for 24 hours. The relative promoter activities were shown. D. K-RTA protein binds to LMP-1 promoter. The LMP1 ISRE was labeled with [α-32P]-dCTP. Equal amounts of partially purified K-RTA, K-RTA-K152E proteins, and purified bovine serum albumin (BSA) were used for EMSA. Specific protein-DNA complex is shown.

**Fig. 5.** LMP-1 inhibits chemically-induced lytic replication of KSHV. A. EBV LMP-1 inhibits the K-RTA expression upon induction of TPA. Vector, LMP-1 or LMP-DM plasmid was transfected into BC3 (KSHV+/EBV-) cells along with CD4 expression plasmid, and the transfected cells were selected by CD4 magnetic beads. After several hours’ recovery, the cells were treated with TPA, and the lysates from enriched and treated cells were used for western blot analysis. The identity of proteins is as shown. B. Recombinant adenovirus expressing LMP-1 inhibits TPA-induced lytic gene expression. Lysates from AdLMP-1, or AdGFP infected BC3 cells were used for western blot analysis. The identity of proteins is as shown.

**Fig. 6.** Down-regulation of LMP-1 enhances KSHV lytic replication. A. siLMP-1 repressed LMP-1 expression. 293T cells were transfected with siLMP-1 and LMP-1 expression plasmid. The cell lysates were used for western blot analysis. The identity of proteins is as shown. B. siLMP-1 repressed LMP-1 expression in dually infected PEL. The concentrated cell lysates from control and siLMP-1-expressing BC1 (KSHV+/EBV+) cells were used for western blot analysis. The identity of proteins is as shown. n.s.: non-specific. C. siLMP-1 enhanced the expression of KSHV lytic proteins. The siLMP-1 expression and its control in BC-1 cells were treated with sodium butyrate for 18 hours. The lysates were used for western blot analysis. D. LMP-1 status in dually-infected PELs. The cell lysates from three PEL lines were used for immunoprecipitation/western blot analyses. BC1, JSC1, and HBL6 are all EBV+/KSHV+ PEL lines. The specific antibodies for immunoprecipitation (IP) and western blot (WB) are indicated. The identity of proteins is as shown.
Fig. 7. LMP-1 and K-RTA are expressed during primary infections. A. KSHV infection induces LMP-1 in EBV latency cells. The KSHV viruses (0.5 or 1 ml) were used to infect 293EBV cells for 24 hours. The cell lysates were used for western blot analyses. The identity of proteins is as shown.

B. K-RTA is involved in the induction of LMP-1. The 293EBV cells were first transfected with vector, siKRTA, and si-p53 expression plasmid. Same amounts of KSHV from same batch were then used to infect these cells a day later. The cell lysates were used for western blot analyses. RNA isolated from these cells was used for semi-quantitative PCR analyses with primers specific to actin and K-RTA. Identity of proteins and RNAs is as shown.

C. KSHV infection induces EBV lytic replication. KSHV was used to infect 293EBV or BZLF1-KO cells. Cell lysates were used for western blot analyses. The same membrane was stripped and reprobed with other antibodies. The identity of proteins is as shown.

D. EBV infection of KSHV induces the expression of K-RTA. Cro6 cells were infected by EBV. The RNA was isolated 24 hours later. RT-PCR was used for detection of K-RTA, LMP-1 as well as actin. The identity of the products is as shown.
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<th>Properties</th>
<th>Growth conditions</th>
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<td>BC-1</td>
<td>EBV+, KSHV+, PEL line (15).</td>
<td>RPMI1640+10% FBS</td>
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<tr>
<td>HBL-6</td>
<td>EBV+, KSHV+, PEL line (33).</td>
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<td>JSC-1</td>
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<tr>
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<td>BC3EBV (cl.10)</td>
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<td>Cro6 EBV (cl.2)</td>
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<td>AGS-BX1g</td>
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A BRLF1-KO (EBV+/KSHV-)

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<tr>
<th>Uninfected</th>
<th>AdGFP</th>
<th>AdGFP</th>
<th>AdRTA</th>
<th>AdRTA</th>
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- LMP-1
- Tubulin
- K-RTA

B BRLF1-KO

- pcDNA3
- K-RTA
- E-RTA

- LMP-1
- E-RTA
- K-RTA

C P3HR1 (EBV+/KSHV-)

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- LMP-1
- K-RTA
- Tubulin
**A**

BRLF1-KO (EBV+/KSHV-)

<table>
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<tr>
<th>GAPDH probe</th>
<th>LMP-1 probe</th>
<th>Yeast RNA</th>
<th>IB4 RNA</th>
<th>pcDNA3</th>
<th>K-RTA</th>
<th>K-RTA-K152E</th>
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</table>

- LMP-1 probe
- LMP-1 RNA
- GAPDH probe
- GAPDH RNA
- (short exposure)

**B**

Relative LMP-1 levels (LMP1/GAPDH)

- pcDNA3
- K-RTA
- K-RTA-K152E

**C**

BRLF1-KO (EBV+/KSHV-)

- pcDNA3
- K-RTA
- K-RTA-K152E

- K-RTAs
- Tubulin
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<tr>
<td></td>
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</table>

- **A**: 
  - K-RTA
  - K8
  - LMPs
  - Tubulin

- **B**: 
  - K-RTA
  - K8
  - LMP-1
  - Tubulin
A

Control | siLMP1
---|---
Vector | LMP1 | LMP1 | LMP1

LMP-1

Tubulin

B

BC-1 | Control | siLMP1 | siLMP1

LMP-1

n.s.

Tubulin

C

siLMP1 | Control
---|---

| 0 | 0.5 | 1 | 0 | 0.5 | 1 | mM Sodium Butyrate

K-RTA

K8

Tubulin

D

IP: LMP-1 | + | + | + | +
IP: STAT-1 | + | - | - | -
Cell lysates | BC1 | BC1 | HBL6 | JSC1

WB: LMP-1

LMP-1

IgG

WB: STAT-1

STAT-1
A

293EBV (EBV+/KSHV-)

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B

293EBV (EBV+/KSHV-)

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<tr>
<th></th>
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C

293-EBV (EBV+/KSHV-)

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D

Cro 6 (EBV-/KSHV+)

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