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Epstein-Barr Virus Inhibits Kaposi's Sarcoma-Associated Herpesvirus Lytic Replication in Primary Effusion Lymphomas^{▽¶}

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The majority of AIDS-associated primary effusion lymphomas (PEL) are latently infected with both Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). PELs harboring two viruses have higher oncogenic potential, suggesting functional interactions between EBV and KSHV. The 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 by EBV of 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.

Both Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8, are members of the human gammaherpesviruses and are 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 Kaposi's sarcoma (16, 18, 60) and is implicated in the pathogenesis of AIDS-associated primary effusion lymphoma (PEL), also called body cavity-based lymphoma, and multicentric Castleman's disease (23, 60, 86).

Like other herpesviruses, EBV and KSHV go through both latency and lytic replication cycles. The 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 is 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 and adhesive, invasive, angiogenic, and antiviral potential (31, 41, 59, 81–83, 87, 90, 94, 95).

Interestingly, the majority of PELs are harboring 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 body cavity-based lymphomas 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 for K-RTA (pCMV50) and the recombinant adenoviruses for green fluorescence protein (GFP) (AdGFP) and K-RTA (AdRTA) were a gift from Byrd Quinlivan (71).

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TABLE 1. Cell lines used in this study and their properties^a

| Cell line | Properties (reference) | Growth conditions |
|-----------------|-------------------------------------------------------------------------------------------------------|-----------------------------------------|
| BC-1 | EBV ⁺ , KSHV ⁺ , PEL line (15) | RPMI 1640 + 10% FBS |
| HBL-6 | EBV ⁺ , KSHV ⁺ , PEL line (33) | RPMI 1640 + 10% FBS |
| JSC-1 | EBV ⁺ , KSHV ⁺ , PEL line (12) | RPMI 1640 + 10% FBS |
| BC3 | EBV ⁻ , KSHV ⁺ , PEL line (5) | RPMI 1640 + 20% FBS |
| BC3-EBV (cl 10) | EBV ⁺ , KSHV ⁺ , PEL line (76) | RPMI 1640 + 20% FBS + G418 (2.5 mg/ml) |
| BCBL-1 | EBV ⁻ , KSHV ⁺ , PEL line (65) | RPMI 1640 + 10% FBS |
| Cro6 | EBV ⁻ , KSHV ⁺ , PEL line (76) | RPMI 1640 + 20% FBS |
| Cro6-EBV (cl 2) | EBV ⁺ , KSHV ⁺ , PEL line (76) | RPMI 1640 + 20% FBS + G418 (1 mg/ml) |
| IB-4 | EBV ⁺ , KSHV ⁻ , EBV-transformed lymphoblastoid cell line (48) | RPMI 1640 + 10% FBS |
| P3HR1 | EBV ⁺ , KSHV ⁻ , Burkitt's lymphoma line; EBNA-2 is deleted in viral genome (2) | RPMI 1640 + 10% FBS |
| DG75 | EBV ⁻ , KSHV ⁻ , Burkitt's lymphoma line (7) | RPMI 1640 + 10% FBS |
| 293T | EBV ⁻ , KSHV ⁻ , fibroblast | DMEM + 10% FBS |
| 293-EBV | EBV ⁺ , KSHV ⁻ , 293 fibroblast-derived wild-type EBV genome (27) | DMEM + 10% FBS + hygromycin (0.5 mg/ml) |
| BRLF-KO | EBV ⁺ , KSHV ⁻ , 293 fibroblast derived; lacking BRLF1 gene in EBV genome (27) | DMEM + 10% FBS + hygromycin (0.5 mg/ml) |
| BZLF1-KO | EBV ⁺ , KSHV ⁻ , 293 fibroblast derived; lacking BZLF1 gene in EBV genome (27) | DMEM + 10% FBS + hygromycin (0.5 mg/ml) |
| AGS-BX1g | EBV ⁺ , KSHV ⁻ ; GFP gene is present in EBV genome (9) | DMEM + 10% FBS + G418 (0.5 mg/ml) |

^a All listed lines are human cell lines. Phenotypes, genotype, properties, and culture conditions are as shown.

The K-RTA K152E mutant is a DNA-binding mutant of K-RTA (91). The recombinant adenovirus for LMP-1 (AdLMP1) was a gift from Stephen Gottschalk and Cliona Rooney (37). The β -galactosidase expression plasmid, pCMV β , was purchased from Clontech. LMP-1 and LMP-DM, which is a mutant LMP-1 failing to initiate signals, have been described previously (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 for the interferon-stimulated response element (ISRE) mutation. The LMP-1 ISRE (5'-AGGAAATGGAAAGG-3') was mutated at the underlined nucleotides for mISRE (5'-AGGGGGTGGGGGGG-3'). The pHp vector was constructed by ligation of the PacI/blunt-XbaI fragment of pAdTrack-HP (96) into the AseI/blunt-XbaI sites of pEGFP-N1 (ClonTech). The sequence 5'-TGGAACGCGACCTTGAGAG-3' was used to clone the small interfering RNA for LMP-1 (siLMP-1) expression plasmid in pHp. The 5'-TGGCGCAAGATGACAAGGG-3' sequence was used for making siRNA for K-RTA. The sequence for p53 has been described previously (96). All constructs were verified by sequence analysis by the UNL Sequencing Facility. The K-RTA antibody was from David Lukac (all figures except Fig. 1). Rabbit polyclonal antibody against K-RTA peptide (amino acids 667 to 691), "ATPAN EVQESGTLYQLHQWRNYFRD," was customer made by Alpha Diagnostic International. This K-RTA antibody was used for Fig. 1. The K8 antibody was

from 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, and STAT-1 monoclonal antibody was from Santa Cruz. Rat monoclonal antibody to KSHV latency-associated nuclear antigen was obtained from ABI. Secondary goat antirat conjugated with cy5 was from Jackson Immuno-Research.

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 (Dyna). The isolated cells were lysed immediately and used for Western blot analysis. However, for chemical treatment, cells were detached from Dynabeads CD4 by incubation for 45 to 60 min at room temperature with 10 μ l of DETACHaBEAD (Dyna). The detached beads were removed by using a magnet separation device. The released cells were washed two to three times with 500 μ l RPMI 1640 plus 10% fetal bovine serum (FBS) and resuspended in RPMI 1640 plus 10% FBS at 5×10^5 cells/ml. Cells were recovered for 2 to 6 h before the addition of chemical reagents. BC1 (EBV⁺ KSHV⁺) cells were transfected with either pHp (control) or pHp-siLMP-1 (siLMP-1) along with a CD4 expression plasmid, and the transfected cells were enriched and cultured in the presence of G418 (1 mg/ml) and 20% FBS for 1 week to make cell lines. The cells were then treated by chemicals for induction of lytic replication.

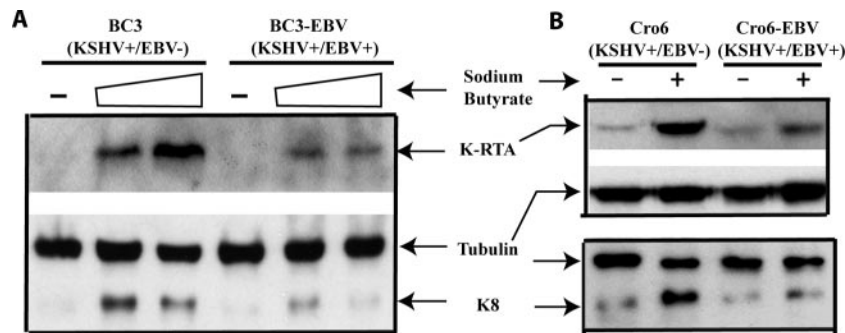


FIG. 1. EBV inhibits KSHV lytic gene expression. (A) EBV inhibits KSHV lytic gene expression in BC3 cells. BC3 cells and their EBV-infected counterparts, BC3-EBV cells, were treated with sodium butyrate (0.5 and 1 mM) for 12 to 16 h. Lysates were separated by 10% SDS-PAGE, transferred, and used for Western blot analysis. The membrane was cut in the middle and probed with different antibodies. Identities of proteins are as shown. (B) EBV inhibits KSHV lytic gene expression in Cro6 cells. Cro6 cells and their EBV-infected counterparts, Cro6-EBV cells, were treated with sodium butyrate for 12 to 16 h. Lysates were used for Western blot analysis. The same membrane was stripped and reprobed with other antibodies. One representative from three experiments is shown. Identities of proteins are as shown.

Preparation of KSHV stocks and in vitro infections. BCBL-1 cells were treated with 12-*O*-tetradecanoyl phorbol-13-acetate (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 $100,000 \times g$ for 1 h. Virion pellets were subsequently resuspended in complete medium (1:100 of the volume of the original supernatants) and were passed through a 0.45- μ m 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 h, 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 30 ng/ml of TPA. Media were replaced with fresh media without TPA and butyrate after 24 h. Media were collected 4 days later with or without concentration and passed through a 0.45- μ m filter. For infection, Cro6 cells were incubated with 0.5 or 1 ml of EBV stock. After incubation at 37°C for 24 h, cells were collected and RNA was isolated for reverse transcription-PCR (RT-PCR) analysis. Infectivity was examined by detecting GFP-positive cells under a UV microscope. Similar results were obtained with concentrated or nonconcentrated EBV viruses (data not shown).

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

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

Induction of KSHV lytic replication. BC3 cells were treated with 5 to 10 ng/ml TPA for 18 to 24 h to induce lytic viral replication. For BC1 cells, 0.5 to 1 mM sodium butyrate was used for 18 to 24 h for the induction of KSHV lytic replication. For experiments shown in Fig. 1, BC3, BC3-EBV (clone [cl] 10), Cro6, and Cro6-EBV (cl 2) cells were treated with sodium butyrate at 0.5 to 1 mM.

Western blot analysis, RNA extraction, RPA, and RT-PCR. Standard Western blot analysis was performed as described previously (92). Total RNA was isolated from cells using the RNeasy Total RNA isolation kit (QIAGEN). RNase protection assays (RPA) were performed with total RNA using the RNase Protection Assay Kit II (Ambion) as described previously (93). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was from U.S. Biochemicals, Inc. The probe for LMP-1 was a gift from 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 (2 μ l) was then PCR amplified with the appropriate primers. PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 30 to 35 cycles of 95°C for 30 s, 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 by 8% polyacrylamide gel electrophoresis (PAGE). Primers used for LMP-1 were 5'-TGA TCATCTTTATCTTCAGAAGAGACCTT-3' and 5'-CCTGTCCGTGCAAAT TCCA-3'; for K-RTA, 5'-CGCAATGCGTTACGTTGTTG-3' and 5'-GCCCG GACTGTTGAATCG-3'; and for actin, 5'-TTCTACAATGAGCTGCGTGT-3' and 5'-GCCAGACAGCACTGTGTTGG-3'.

Cell lysates and immunoprecipitation. Cells (10^8) were collected by centrifugation and were resuspended in 1 ml of extraction buffer (0.5% NP-40, 0.3 M NaCl, 0.1 mM EDTA, 20 mM HEPES [7.4], 1 mM dithiothreitol, and 10% glycerol), supplemented with complete protease inhibitors (Roche). The cell suspension was kept in 4°C with gentle agitation. Insoluble material was pelleted at $14,000 \times g$ 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 μ l of protein G Sepharose (Pharmacia), and the sample was incubated overnight at 4°C with continuous mixing. Protein immunocomplexes were isolated by centrifugation, and beads were washed three times with extraction buffer. Immunoprecipitated proteins were resuspended in sodium dodecyl sulfate (SDS)-PAGE loading buffer and analyzed by SDS-PAGE, and Western blot analyses were employed with specific antibodies.

RESULTS

EBV inhibits lytic replication of KSHV. Because the majority of the current studies on KSHV and EBV use singly infected cells as a model system, the potential effects of EBV and KSHV on each other's 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 those for EBV nuclear antigen 1 (EBNA-1), -2, -3, -4, and -6, LMP1, -2A, and -2B, and EBV-encoded small RNAs, was examined with these isogenic cell lines. All EBV-infected clones of the PEL cells expressed EBNA-1, EBV-encoded small RNA 1, and LMP2A. EBNA2 to -6 were not expressed in any of the converted lines. The expression of LMP-1 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 (76). We now have shown that LMP-1 is expressed in both BC3-EBV and Cro6-EBV lines in an RT-PCR assay (see Fig. S1 in the supplemental material). 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 recombinant EBV containing GFP was used for the establishment of isogenic lines, expression of GFP could be used to identify these EBV-positive cells. We found that these EBV convertants were 100% positive for KSHV as determined by the expression of KSHV latency-associated nuclear antigen (also called ORF73) (see Fig. S2A in the supplemental material) and 65% and 85% GFP positive for BC3-EBV and Cro6-EBV, respectively (see Fig. S2B in the supplemental material). Thus, BC3-EBV and Cro6-EBV had 65 and 85% cells, respectively, that were 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 the same amounts of sodium butyrate, and KSHV lytic gene expression was examined by Western blot analyses. As shown in Fig. 1, expression of the K-RTA and K8 genes was 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 expression of EBV LMP-1 protein. The EBV LMP-1 promoter region has three kinds of *cis* elements through which K-RTA could possibly regulate: RBP-J κ binding sequence (RBP-J κ), ISRE, and gamma interferon activation sequence (GAS) (40, 51, 52, 91) (see 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). The EBV BRLF1 gene product (or E-RTA) has been shown to

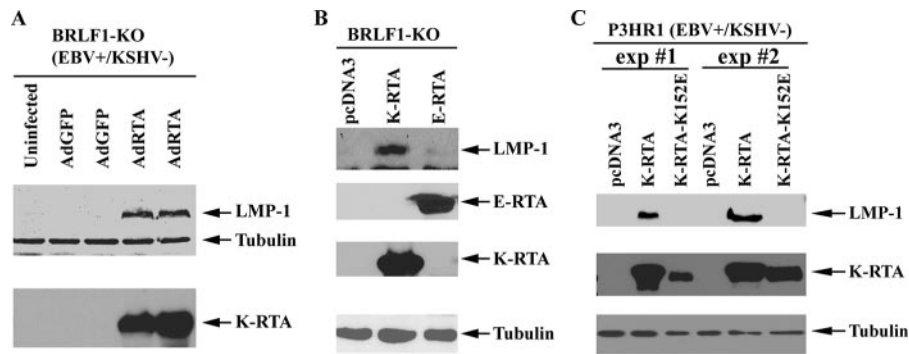


FIG. 2. KSHV K-RTA induces expression of EBV LMP-1. (A) Recombinant adenovirus containing K-RTA induces expression of LMP-1. Lysates from uninfected or AdRTA- or AdGFP-infected BRLF1-KO cells were used for Western blot analysis. BRLF1-KO contains EBV BRLF1-null genome. The same membrane was stripped and reprobed with other antibodies. Identities of proteins are as shown. (B) Transfection of K-RTA induces 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) 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 the CD4 expression plasmid, and transfected cells were selected by CD4 magnetic beads. P3HR1 is an EBV⁺ line with EBNA-2 deletion in the genome. Lysates from enriched cells were used for Western blot analysis. The same membrane was stripped and reprobed with other antibodies. Identities of proteins are as shown.

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 infection 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 ex-

amined the effect of K-RTA in transient-transfection assays. An expression plasmid of K-RTA or the 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. 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

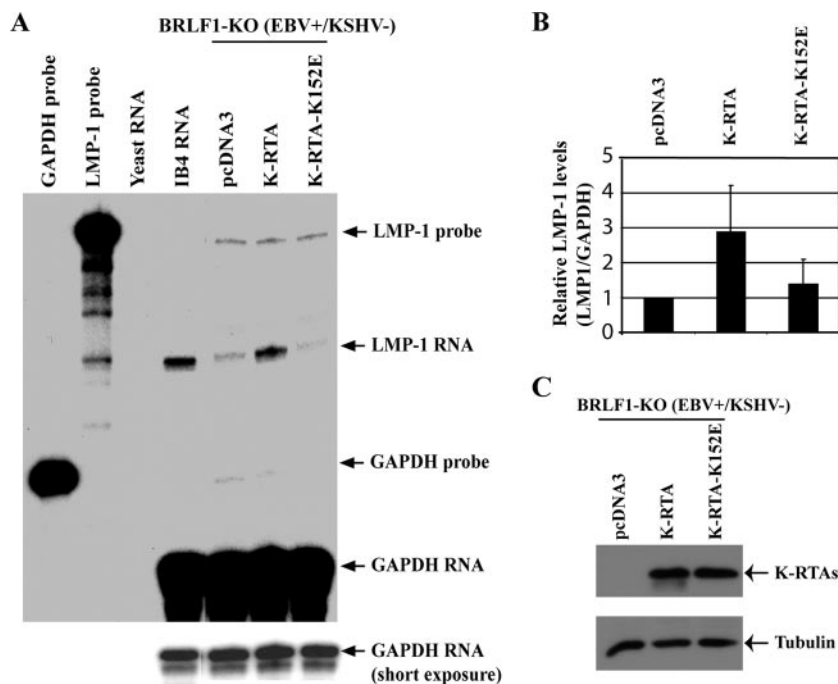


FIG. 3. K-RTA induces expression of LMP-1 RNA. (A) K-RTA induces LMP-1 RNA. LMP-1 and GAPDH probes were labeled with [α -³²P]UTP and used for RPA. Yeast RNA, RNA from IB4 cells (positive control), or RNAs from BRLF1-KO cells transfected with pcDNA3, K-RTA, or K-RTA-K152E, respectively, were used for 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. Identities of proteins are as shown.

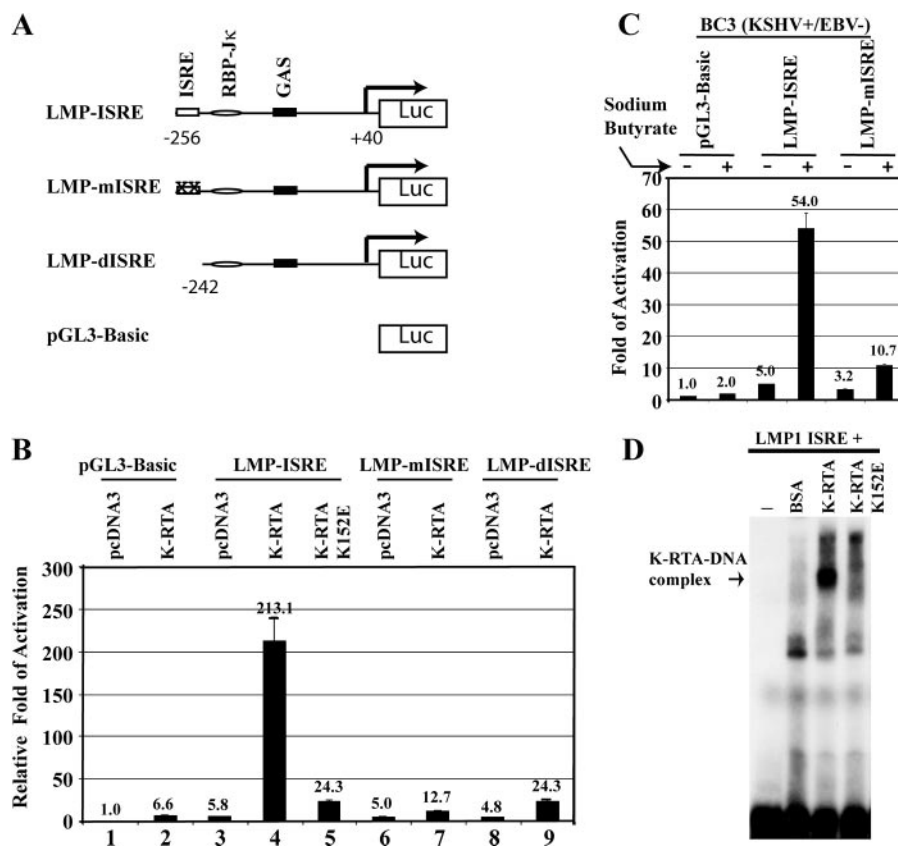


FIG. 4. KSHV K-RTA activates EBV LMP-1 promoters. (A) Schematic diagram of EBV LMP-1 promoter reporter constructs. RNA start site is shown. The drawing is not to scale. (B) K-RTA activates EBV LMP-1 promoter reporter constructs. 293T cells were transfected with various reporter constructs along with the CMV- β -gal, K-RTA, or K-RTA-K152E expression plasmid as shown on the top. Luciferase activity was normalized to β -galactosidase activity. The n -fold activation of each promoter construct is shown with standard deviation. One representative of five 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 (3 mM) for 24 h. The relative promoter activities are 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 and K-RTA-K152E proteins and purified bovine serum albumin were used for EMSA. Specific protein-DNA complex is shown.

the 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; also 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 the LMP-1 protein by K-RTA is independent of EBNA-2. Because 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, which in turn activates LMP-1. P3HR1 (EBV⁺ KSHV⁻) is a Burkitt's lymphoma 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 details). 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 the 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 the LMP-1 protein in BRLF1-KO cells (data not shown). Because K-RTA-K152E has a 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. K-RTA or its mutant K-RTA-K152E was transfected into BRLF1-KO cells. Total RNA was isolated from transfected cells 24 h later. RPA were used for detection of LMP-1 RNA. K-RTA-transfected cells have higher LMP-1 RNA levels than vector- or K-RTA-K152E-transfected cells (Fig. 3A and B). 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 transacti-

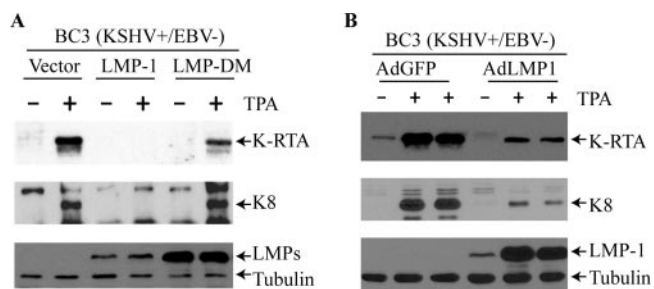


FIG. 5. LMP-1 inhibits chemically induced lytic replication of KSHV. (A) EBV LMP-1 inhibits K-RTA expression upon induction of TPA. Vector, LMP-1, or LMP-DM plasmid was transfected into BC3 (KSHV⁺ EBV⁻) cells along with CD4 expression plasmid, and transfected cells were selected by CD4 magnetic beads. After several hours' recovery, the cells were treated with TPA, and lysates from enriched and treated cells were used for Western blot analysis. Identities of proteins are 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. Identities of proteins are as shown.

vation: RBP-J κ , 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 (see Fig. S3A in the supplemental material). As shown in Fig. 4B, K-RTA was able to activate empty pGL3basic two- to sixfold under our conditions (columns 1 and 2), which is a background issue. The LMP-ISRE-luc reporter was strongly activated by K-RTA (columns 3 and 4), but the point mutations or deletion of ISRE crippled activation by K-RTA (columns 6 to 9). In agreement with the data shown in Fig. 2C and 3A, the K-RTA-K152E mutant failed to activate LMP-1 promoter reporter constructs (Fig. 4, column 5). Because LMP-ISRE-luc contains ISRE, RBP-J κ , 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 two- to threefold activations were observed when pGL3-basic or LMP-mISRE-luc was used for transfection (Fig. 4C). Moreover, in DG75 (EBV⁻ KSHV⁻) cells, sodium butyrate could not activate the 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 (see Fig. S3B in the supplemental material). 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 the ISRE (91), it is expected that K-RTA could bind to the LMP-1 ISRE. We first tested if the LMP-1 ISRE was able to 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 the LMP-1

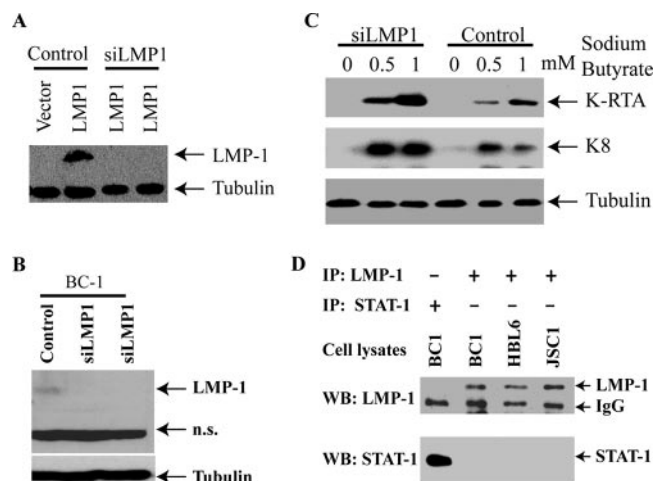


FIG. 6. Down-regulation of LMP-1 enhances KSHV lytic replication. (A) siLMP-1 repressed LMP-1 expression. 293T cells were transfected with siLMP-1 and LMP-1 expression plasmid. Cell lysates were used for Western blot analysis. Identities of proteins are as shown. (B) siLMP-1 repressed LMP-1 expression in dually infected PEL. Concentrated cell lysates from control and siLMP-1-expressing BC1 (KSHV⁺ EBV⁺) cells were used for Western blot analysis. Identities of proteins are as shown. n.s., nonspecific. (C) siLMP-1 enhanced expression of KSHV lytic proteins. siLMP-1 expression and its control in BC-1 cells were treated with sodium butyrate for 18 h. Lysates were used for Western blot analysis. (D) LMP-1 status in dually infected PELs. 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 IP and Western blotting (WB) are indicated. Identities of proteins are as shown.

ISRE probe. The purification process was done according to the method of our previous report, and partially purified K-RTA has the expected properties (91). The same amounts of partially purified proteins were used for EMSA. The binding of K-RTA but not K-RTA K152E to the LMP1 ISRE probe was observed (Fig. 4D). Some bands regularly appeared with our purified K-RTAs. Purified bovine serum albumin was used to detect these nonspecific binding patterns. In addition, cold LMP-ISRE was able to compete for binding to K-RTA, and the K-RTA-DNA complex could be supershifted by the use of K-RTA-specific antibody (see Fig. S3C in the supplemental material). The purified proteins have similar purities as determined by Coomassie blue staining (see Fig. S3D in the supplemental material). 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-immunoglobulin M 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 details). 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). Fur-

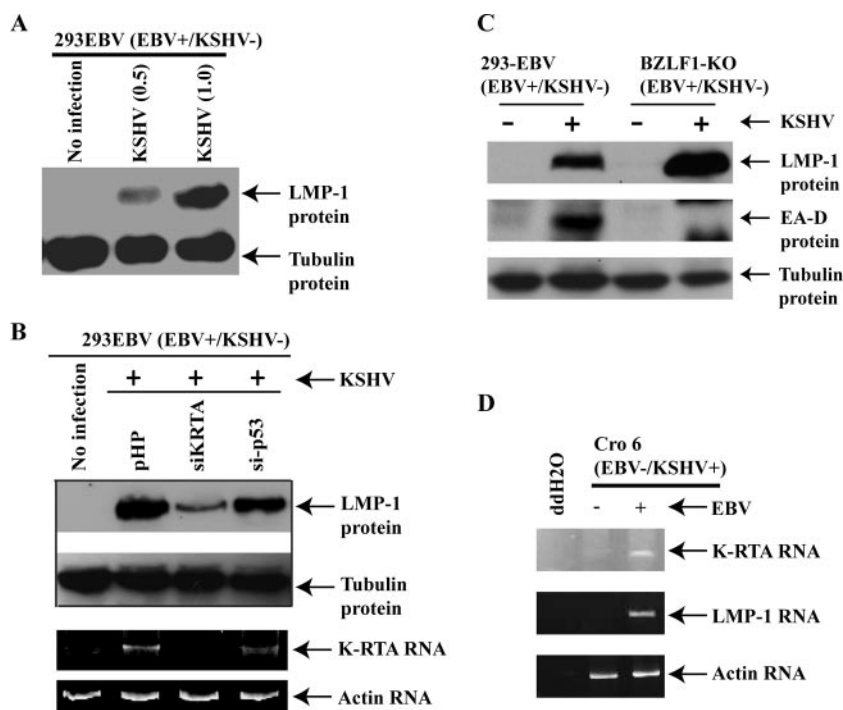


FIG. 7. LMP-1 and K-RTA are expressed during primary infections. (A) KSHV infection induces LMP-1 in EBV latency cells. The KSHV viruses (0.5 or 1 ml) were used to infect 293-EBV cells for 24 h. Cell lysates were used for Western blot analyses. Identities of proteins are as shown. (B) K-RTA is involved in induction of LMP-1. 293-EBV cells were first transfected with vector, siRNA for K-RTA (siKRTA), or si-p53 expression plasmid. The same amounts of KSHV from the same batch were then used to infect these cells a day later. Cell lysates were used for Western blot analyses. RNA isolated from these cells was used for semiquantitative PCR analyses with primers specific to actin and K-RTA. Identities of proteins and RNAs are as shown. (C) KSHV infection induces EBV lytic replication. KSHV was used to infect 293-EBV or BZLF1-KO cells. Cell lysates were used for Western blot analyses. The same membrane was stripped and reprobed with other antibodies. Identities of proteins are as shown. (D) EBV infection of KSHV induces expression of K-RTA. Cro6 cells were infected by EBV. RNA was isolated 24 h later. RT-PCR was used for detection of K-RTA, LMP-1, and actin. Identities of products are as shown.

thermore, 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 was generated and was able to suppress the expression of LMP-1 significantly in transient-transfection assays with 293T cells (Fig. 6A). siLMP-1 was used to generate a stable cell line with BC1 cells, 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 had higher expression of K-RTA and K8 than control cells (Fig. 6C). The 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 with 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 of 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 for choosing 293-EBV is that KSHV apparently infects fibroblasts efficiently (49). Given the fact that infection by KSHV of B cells is inefficient (19, 44), infection of 293-EBV 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 h 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 use of 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 293-EBV 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 infection by EBV of 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 singly infected PELs 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 induces EBV lytic replication in EBV singly 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 the RNA level (Fig. 2 and 3). K-RTA activates LMP-1 promoter reporter constructs and binds to the 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 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 in 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 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 the E-RTA gene (3). NF- κ B has been shown to be a negative regulator of gammaherpesvirus lytic replication (10). LMP-1 but not LMP-DM induces NF- κ B activity (94). Because LMP-DM failed to inhibit KSHV lytic replication, LMP-1-mediated NF- κ B activation is apparently associated with the inhibition of KSHV lytic replication. Thus, the finding that LMP-1 inhibits the lytic replication process of KSHV might be in agreement with previously identified mechanisms for LMP-1 control of 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 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 during either 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 primary infection may inhibit both EBV and KSHV lytic replications and promote establishment of latency for both viruses during primary infections. Of note is that the LMP-1 protein may be present in EBV virions 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.

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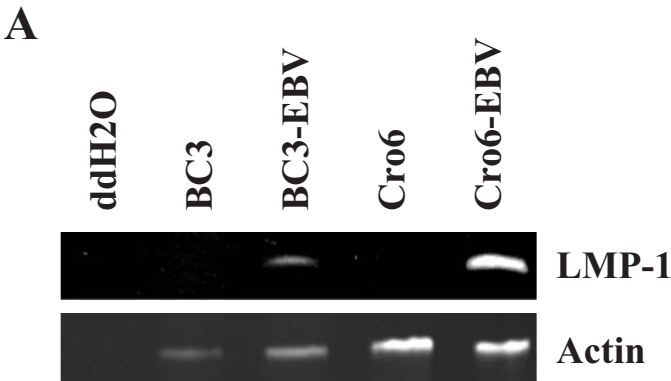
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Expression of EBV LMP-1 in dually infected PELs. The total RNAs from indicated cell lines were isolated, digested with DNase, and reverse transcribed. RT-PCR was used to detect the expression of LMP-1. The identity of RNAs is as shown.

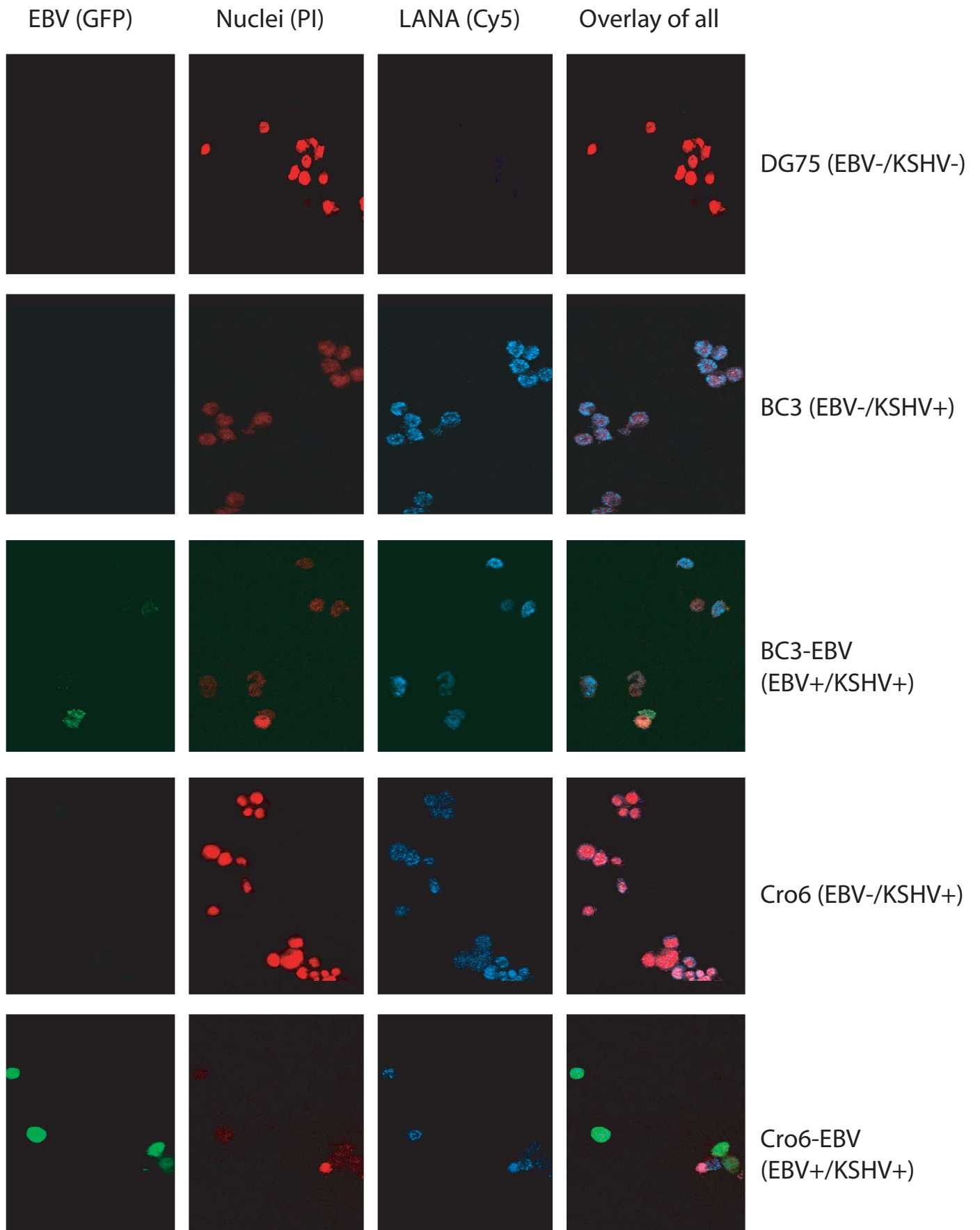
Supplementary Figure 2: Detection of KSHV and EBV in PELs. (A) Detection of KSHV LANA expression in PELs. Growing PEL cells were washed, fixed, and permeabilized. The cells were then stained with LANA antibody and secondary antibody conjugated with Cy5, and counterstained with propidium iodide (PI). Cells were visualized by a confocal microscope. **(B) Detection the percentage of GFP-positive cells.** Growing PEL cells were washed twice with 1x PBS, and subjected to fluorescence-activated cell sorting (FACS) analysis. Live cells were gated and assayed for GFP intensity. The GFP profiles of the cells were plotted, and the percentage of GFP-positive cells was calculated. Solid lines: BC3 or Cro6 cells; dashed lines: BC3-EBV or Cro6-EBV.

Supplementary Figure 3: (A) Expression of RTA and its mutant in transfected cells. Lysates from pcDNA3, RTA, or RTA-K152E transfected 293T cells were used for western blot analysis with K-RTA and tubulin antibodies. Same membrane was used for both antibodies. The identity of proteins is as shown. **(B) Expression of RTA in BC3 cells.** Lysates from sodium butyrate treated BC3 cells were used for western blot analysis with K-RTA and tubulin antibodies. The identity of proteins is as shown. **(C) K-RTA binds to LMP1 ISRE.** The LMP1 ISRE probe was labeled with [α -³²P]-dCTP. Cold probe, LMP-ISRE, was added at 50-fold molar excess over hot probe. Purified bovine serum albumin (BSA) was used as negative controls. Normal rabbit serum (NRS), Rabbit polyclonal Anti-RTA or IRF-1 were used for super shift analyses. Specific protein-DNA complexes are as shown. ss: super-shifted complex. **(D) The purity of purified K-RTAs.** Coomassie blue staining of the partially purified *E. coli*-derived K-RTA and its mutant K-RTA K152E proteins. The identities of these proteins were confirmed by western blot (data not shown).

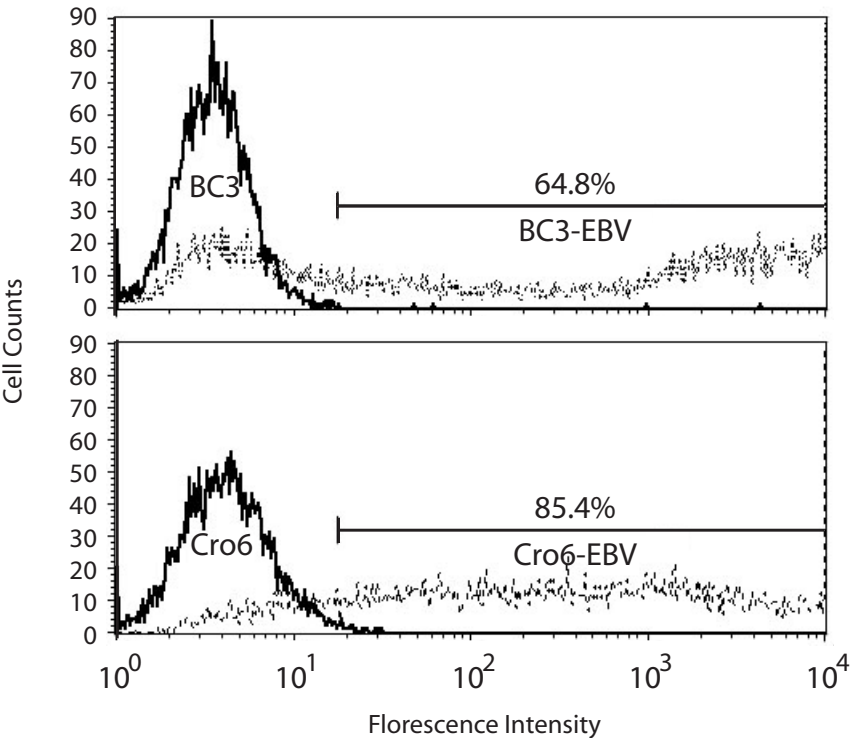
Supplementary Figure 1



Supplementary Figure 2A

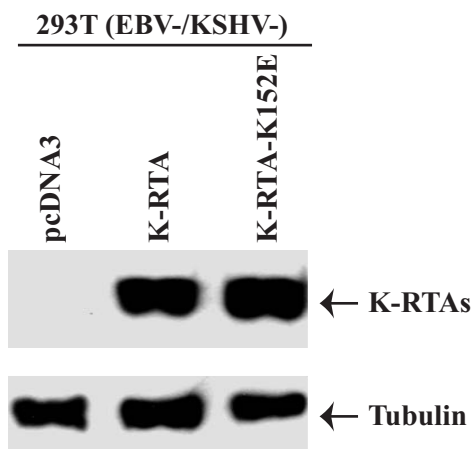


Supplementary Figure 2B

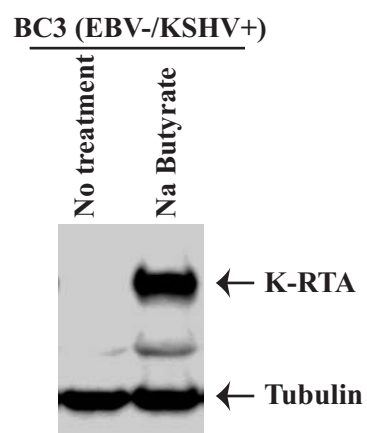


Supplementary Figure 3

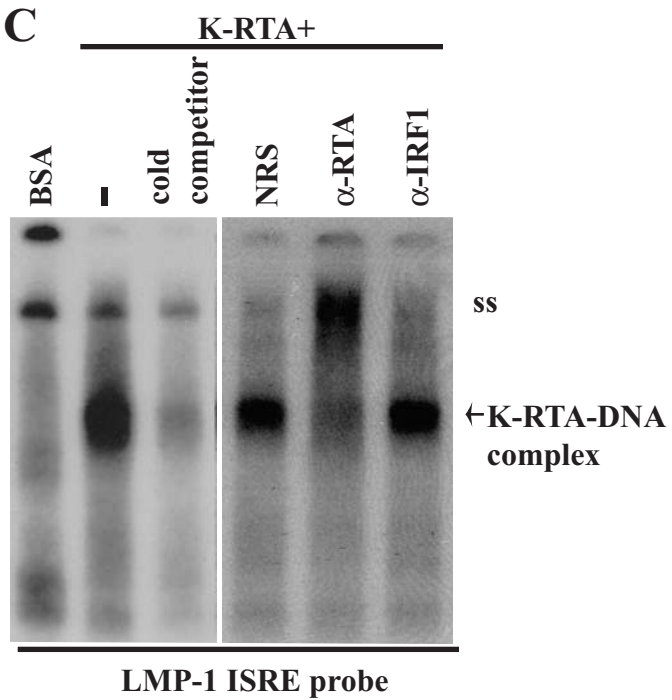
A



B



C



D

