Inhibition of KAP1 Enhances Hypoxia-Induced Kaposi’s Sarcoma-Associated Herpesvirus Reactivation through RBP-Jκ

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ABSTRACT

Hypoxia-inducible factor 1α (HIF-1α) has been frequently implicated in many cancers as well as viral pathogenesis. Kaposi’s sarcoma-associated herpesvirus (KSHV) is linked to several human malignancies. It can stabilize HIF-1α during latent infection and undergoes lytic replication in response to hypoxic stress. However, the mechanism by which KSHV controls its latent and lytic life cycle through the deregulation of HIF-1α is not fully understood. Our previous studies showed that the hypoxia-sensitive chromatin remodeler KAP1 was targeted by the KSHV-encoded latency-associated nuclear antigen (LANA) to repress expression of the major lytic replication and transcriptional activator (RTA). Here we further report that an RNA interference-based knockdown of KAP1 in KSHV-infected primary effusion lymphoma (PEL) cells disrupted viral episome stability and abrogated sub-G1/G0 arrest of the cell cycle while increasing the efficiency of KSHV lytic reactivation by hypoxia or using the chemical 12-O-tetradecanoylphorbol-13-acetate (TPA) or sodium butyrate (NaB). Moreover, KSHV genome-wide screening revealed that four hypoxia-responsive clusters have a high concurrence of both RBP-Jκ/HRE within the same gene promoter and are tightly associated with KAP1. Inhibition of KAP1 greatly enhanced the association of RBP-Jκ with the HIF-1α complex for driving RTA expression not only in normoxia but also in hypoxia. These results suggest that both KAP1 and the concurrence of RBS+HRE within the RTA promoter are essential for KSHV latency and hypoxia-induced lytic reactivation.

IMPORTANCE

Kaposi’s sarcoma-associated herpesvirus (KSHV), a DNA tumor virus, is an etiological agent linked to several human malignancies, including Kaposi’s sarcoma (KS) and primary effusion lymphoma (PEL). HIF-1α, a key hypoxia-inducible factor, is frequently elevated in KSHV latently infected tumor cells and contributes to KSHV lytic replication in hypoxia. The molecular mechanisms of how KSHV controls the latent and lytic life cycle through deregulating HIF-1α remain unclear. In this study, we found that inhibition of hypoxia-sensitive chromatin remodeler KAP1 in KSHV-infected PEL cells leads to a loss of viral genome and increases its sensitivity to hypoxic stress, leading to KSHV lytic reactivation. Importantly, we also found that four hypoxia-responsive clusters within the KSHV genome contain a high concurrence of RBP-Jκ (a key cellular regulator involved in Notch signaling) and HIF-1α binding sites. These sites are also tightly associated with KAP1. This discovery implies that KAP1, RBP-Jκ, and HIF-1α play an essential role in KSHV pathogenesis through subtle cross talk which is dependent on the oxygen levels in the infected cells.

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a large DNA tumor virus implicated in the etiology of many human cancers: Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL), and a subset of multicentric Castleman’s disease (MCD) (1, 2). Like all herpesviruses, KSHV establishes a similar paradigm for viral cancers, which results in a productive cycle or latent replication. During latency, the latency-associated nuclear antigen (LANA) encoded by open reading frame 73 (ORF73) is the dominant latent protein, with multiple functions for establishment of latent infection (3–5). The majority of the viral genes are silenced, and the viral genome persists in host cells as an episome with a condensed chromatin state. In lytic replication, the replication and transcriptional activator (RTA) encoded by ORF50 is essential for initiation of viral lytic replication (6–9). Reactivation of KSHV from latency leads to increased risk of developing KS and PEL (6, 8). The switch from KSHV latent to lytic replication can be initiated by specific intracellular signals or extracellular stimuli, including hypoxia (10, 11), 12-O-tetradecanoylphorbol-13-ace...
lated in both KSHV latently infected cells and KS patient tissues in
tion of Ser824, SUMOylation of KAP1 at Lys554, Lys779, and Lys804
response to genotoxic stress. While antagonistic to phosphoryla-

viral chromatin remodeling from the heterochromatin to the eu-
ger proteins (13,15, 16). KAP1 can coordinate the assembly of
a universal transcriptional corepressor, is a member of the family
findings
-
RCGTGC-3

KAP1 (also known as TRIM28 or TIF1β), initially identified as
a universal transcriptional corepressor, is a member of the family of
KRAB (Kruppel-associated box) domain-containing zinc fing-
proteins (13, 15, 16). KAP1 can coordinate the assembly of
several chromatin-remodeling proteins, such as histone deacetylase
compound (HDAC), the histone methyltransferases (e.g., SETDB1),
and HP1, to create an epigenetically stable and heritable hetero-

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MATERIALS AND METHODS

Antibodies. KAP1 (20C1) antibodies were purchased from Abcam (Cam-
bridge, MA). HIF-1α antibodies were from BD Transduction Labora-
tory (San Jose, CA). Sin3A (AK-11) and PARP1 (F2) were purchased from
Santa Cruz Biotech. Inc. (Santa Cruz, CA). Glyceraldehyde-3-phosphate
der hydrogenase (GAPDH; G8140-01) antibodies were from United States
Biological Inc. (Swampscott, MA). Mouse monoclonal antibodies
against LANA, RTA, and RBP-Jκ (BW9369) were used as described previ-
ously (31).

Cell culture and hypoxic incubation. KSHV-positive (BC3 and
BCBL1) B lymphoma cells were cultured in RPMI medium supplemented
with 10% fetal bovine serum (FBS; HyClone). HEK293 cells were cultured
in Dulbecco modified Eagle medium (DMEM) supplemented with 10%
(FBS; HyClone). Cells were grown in a humidified atmosphere at 37°C and
gas tensions of 21% O2–5% CO2 for normoxic incubation and 1% O2–5%
CO2 for hypoxic incubation as described previously (32).

Stable RNAi-expressing cell line production and transduction. The
KAP1 short hairpin RNA (shRNA) sequence (5'-GCGATGAACCCCTTGTGCCTG-3'), Sin3A shRNA sequence (5'-CAACCTGCTAGAAAGGTGATTCTTGCT-3'), and control sequence (5'-TGCGGCTGCTGACTCACA-3'; nontargeting sequence) were individually inserted into the pGIPz vector
according to the manufacturer’s instructions (Clontech). The pGIPz vec-
tor containing shRNA sequence was cotransfected with lentivirus packag-
ing plasmids (Rev, vesicular stomatitis virus G protein [VSVG], and gp)
into CoreT cells by the calcium phosphate method to generate virus. The
packaged viruses were used to individually transduce target cells (BC3 and
BCBL1) and selected using 2 μg/ml of puromycin. The RNA interference
(RNAi) efficiency was assessed by Western blot analysis with specific
KAP1 or Sin3A antibodies.

Flow cytometry of cell cycle. Cells were harvested, washed in ice-cold
phosphate-buffered saline (PBS), and fixed in cold methanol-acetone (50/
50). Cells were stained with PBS containing 40 μg/ml of propidium iodide
(P1), 200 μg/ml of RNase A (Sigma), and 0.05% Triton X-100 for 1 h
at room temperature in the dark. Cell cycle profiles of stained cells were
analyzed using FACSscan (BD Biosciences, Foster, CA) and FlowJo soft-
ware.

Quantitative PCR. Total RNA from cells was extracted using TRIzol,
and cDNA was made with a Superscript II reverse transcription kit (In-
vitrogen, Inc., Carlsbad, CA). The primers used for real-time PCR are
shown in Table 1. The cDNA was amplified using 10 μl of master mix
from the DyNAmo SYBR green quantitative real-time PCR kit (M1 Re-
search, Inc.), a 1 μM concentration of each primer, and 2 μl of the cDNA
product in a 20-μl total volume. Thirty cycles of 1 min at 94°C, 30 s
at 55°C, and 40 s at 72°C were followed by 10 min at 72°C in a Step-One
thermocycler (Applied Biosystems Inc.). A melting-curve analysis was
performed to verify the specificities of the amplified products. The values
for the relative levels of change were calculated by the threshold cycle
(ΔΔCT) method, and samples were tested in triplicates

Extraction and quantitation of KSHV episomal DNA. Total DNA was
extracted by lysis buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10
mM EDTA, 1% SDS), followed by proteinase K digestion. Relative numbers of KSHV episomal copies were calculated by quantitative PCR
(qPCR) amplification of the terminal repeats (TR) or K8 gene primers as
previously described (33). For intracellular episomal DNA, GAPDH con-
tent in each sample was first amplified as an internal control to normalize
DNA input for TR amplification. For extracellular viral DNA, the viral

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TABLE 1 Primers used for qPCR

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<td>Antisense primer: 5'-TTTCGAGGAGGAGGTGGTGTA-3'</td>
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<tr>
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<td>Antisense primer: 5'-AAGGACCATCAGAGGATTAG-3'</td>
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0.2% fish skin gelatin (G-7765; Sigma, Chicago, IL) and 0.2% Triton X-100 for 5 min, followed by primary and secondary antibody staining. DNA was counterstained with 4',6'-diamidino-2-phenylindole (DAPI), and coverslips were mounted with p-phenylenediamine. Cells were visualized with a Fluoview FV3000 (Olympus Inc., Melville, NY) confocal microscope.

ChIP. Chromatin immunoprecipitation (ChIP) experiments were done as previously described (34, 35), with some modifications. Cells (3 × 10^8) were cross-linked with 1.1% (vol/vol) formaldehyde, 100 mM NaCl, 0.5 mM EGTA, and 50 mM Tris-HCl (pH 8.0) in growth medium at 37°C for 10 min and then at 4°C for 50 min. Formaldehyde was quenched by adding 0.05 volume of 2.5 M glycine. Fixed cells were washed with PBS and incubated for 15 min in 15 ml of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5 mM EGTA, and 0.25% (vol/vol) Triton X-100, followed by 15 min in 15 ml of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, and 200 mM NaCl, and finally sonicated in 1 ml of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% (wt/vol) SDS plus 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 µg/ml of aprotinin, leupeptin, and pepstatin to an average fragment size of 300 to 500 bp. Solubilized chromatin extracts were clarified by centrifugation at 12,000 × g and diluted to 6 optical density units (optical density at 260 nm [OD260]/ml) in IP buffer (140 mM NaCl, 1% [wt/vol] Triton X-100, 0.1% [wt/vol] sodium deoxycholate, 1 mM PMSF, 100 µg/ml of salmon sperm DNA, and 100 µg/ml of bovine serum albumin [BSA]), preincubated for 1 h at 4°C with 10 µl of 50% (vol/vol) protein A-agarose with normal mouse or rabbit serum, reconstituted in PBS, and washed several times in IP buffer. Aliquots (600 µl) were incubated with 20 µg of each specific antibody or IgG control overnight at 4°C. Immune complexes were separated into bound and unbound complexes with protein A-agarose, and cross-links were reversed by treatment at 65°C overnight. After treatment with RNase A and proteinase K, samples were extracted once with phenol-chloroform, and the DNA was precipitated with 2 volumes of ethanol plus 10 µg of glycogen as a carrier (Roche). Precipitated DNA was pelleted, washed once with 70% ethanol, dried, and resuspended in 100 µl of water. The ChIP DNA and 10% input were amplified by qPCR using specific primers with amplifications of 150 to 200 bp.

Preparation of nuclear extracts. Cells were resuspended in 4 times the volume of the pellet in NE buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl2 with protease inhibitors) after a cold PBS wash, incubated on ice for 1 h, and then transferred to a prechilled Dounce homogenizer and homogenized with 25 strokes. Homogenized samples were transferred to Eppendorf tubes and spun at 2,000 rpm for 5 min at 4°C. The supernatant was aspirated and the pellet resuspended in 2 times the volume of NE buffer B (20 mM HEPES [pH 7.9], 10% glycrol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA with protease inhibitors), followed by incubation on ice for 30 min and centrifugation at 13,000 rpm for 20 min at 4°C. The supernatants were transferred to fresh Eppendorf tubes, and an equal volume of NE buffer C (20 mM HEPES [pH 7.9], 30% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA with protease inhibitors) was added. This was then aliquoted, and the samples were snap-frozen at −80°C before use.

In vitro DNA binding affinity assay. Two complementary 5′-biotinylated oligonucleotides with or without the HIF-1α binding site (wild type, 5′-GGATTCACAATTCGGCCAGCGGT-3′; mutant, 5′-GGATTCACAATTCGGCCAGCGGT-3′; underline indicates HIF-1α binding site) were annealed to be double-stranded DNA and coupled to streptavidin-conjugated agarose beads. Per sample, 3 µg of biotinylated oligonucleotide was incubated with 40 µl of 50 µl of streptavidin-conjugated agarose bead slurry in a total volume of 100 µl of a lysis buffer comprised of 50 mM Tris-HCl (pH 8.0), 15 mM NaCl, 0.1 mM EDTA, 10% glycerol, 10 mM N-ethylmaleimide (NEM), 1 mM PMSF, 1 mM diithiothreitol (DTT), 1 ng/ml of aprotinin, 1 µg/ml of leupeptin, and 1 µg/ml of pepstatin for 2 h at 4°C. Cell nuclear extracts (400 µg) were incubated with 40 µl of DNA-coupled agarose beads in lysis buffer at a total volume of 500 µl for 3 h at 4°C. The precipitated complexes were washed three times with lysis.
buffer. Purified DNA-binding proteins were boiled in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting. A 5% input amount of biotinylated DNA oligonucleotide was verified by dot blotting.

Dual-luciferase reporter assay. Firefly luciferase reporter plasmid pRta-luc with HRE or RBS/H11001 HRE was used to detect the effect of KAP1 knockdown on RTA gene expression in normoxia and hypoxia. The luciferase reporter assays were performed as described previously (36). Thymidine kinase (TK) promoter-driven Renilla luciferase was used as a control to normalize the transfection efficiency.

Statistical analysis. Statistical significance of differences between means of at least 3 experiments was determined using Student’s t test (P values indicated accordingly in figure legends or text below). Error bars represent positive and negative standard errors of the means (SEM).

RESULTS

Establishment of PEL cells with constitutive knockdown of KAP1. Previous studies have shown that LANA associated with KAP1 and Sin3A through its SUMO2-interacting motif (LANA<sub>SIM</sub>) to maintain KSHV latency (26). To further determine if inhibition of KAP1 or Sin3A has effects similar to those of the LANA<sub>SIM</sub> deletion on the long-term maintenance of the KSHV episome during latency, we individually generated 3 clones each of KSHV-infected PEL cell lines BC3 and BCBL1 with constitutive knockdown of KAP1 or Sin3A or a nonspecific control by using lentivirus carrying shRNA (shCtrl) from panel A were used to detect the relative intracellular viral episome copy number by quantitative PCR with TR as a target. GAPDH was used as an internal control. The results are presented as the average relative fold compared with parental cells from 3 independent knockdown clones from panel A. Double asterisks indicate a P value less than 0.05.

Inhibition of KAP1 reduced KSHV episomal copy number but facilitated lytic reactivation induced by TPA-NaB or hypoxia. To investigate whether the inhibition of KAP1 or Sin3A disrupts KSHV episome stability, we checked the copy number of KSHV episomal DNAs in these KAP1 or Sin3A knocked-down cell lines by quantitative PCR analysis. The results showed that both KAP1 and Sin3A knockdown markedly reduced the viral episome copy number compared with parental or knockdown control (Fig. 1B). In view of the fact that both KAP1 and Sin3A are critical chromatin silencers for KSHV latency, we wanted to determine if the reduced expression of KAP1 or Sin3A would enhance the relative efficiency of viral reactivation induced by hypoxia; although constitutive knockdown of KAP1 or Sin3A resulted in a lower viral episomal DNA copy number in the parental PEL cells. To do so, we exposed KAP1 or Sin3A knockdown cell lines to hypoxia at different time points (0, 18, and 36 h), followed by quantitation of progeny virion production. The chemical stimuli TPA and NaB were used as a positive control. The results revealed that KAP1 or Sin3A knockdown in PEL cells consistently led to a relatively higher response to hypoxic stress within 36 h of exposure compared to parental or control knockdown (Fig. 2A, right side; sim-
ilar results were obtained with BCBL1 knockdown cells). Intrigu-
ingly, a similar pattern was observed in the KAP1 or Sin3A knockdown cells in response to TPA-NaB treatment, although there was a slightly higher response in the TPA-NaB group than that in the hypoxia group after 18 h treatment (Fig. 2A; similar results were obtained for the BCBL1 knockdown cells).

To determine if the higher response to stimuli leads to more infectious virion production of KAP1 or Sin3A knockdown cells, we performed PBMC primary infection in vitro using purified virions from the same amount of parentally derived PEL cells induced for 36 h. The results consistently showed that about 87.5% of the PBMCs were infected by virions from the KAP1 knockdown cells, compared to 42% and 40.1% from parental and the control knockdown cells, respectively (Fig. 2B). In contrast, although Sin3A knockdown cells showed a relatively higher sensitivity to lytic replication stimuli, there was much less production of infectious virions (17.3%) due to a smaller amount of viral episomes retained. This indicates that KAP1 is involved in both TPA-NaB- and hypoxia-mediated signaling pathways.

To further address the effects of KAP1 and Sin3A inhibition on TPA-NaB- and hypoxia-mediated lytic reactivation, we determined the protein levels of LANA and RTA expression by Western blot analysis. Unexpectedly, we observed that TPA-NaB treatment led to enhanced RTA expression along with reduced LANA expression, whereas hypoxia treatment not only enhanced RTA expression but also moderately enhanced LANA expression (Fig. 3A, compare lanes 1, 4, and 7). In contrast, KAP1 knockdown efficiently blocked these effects on LANA levels induced by TPA-NaB or hypoxia, while Sin3A knockdown showed similarly less LANA expression and almost undetectable levels for RTA after TPA-NaB or hypoxia treatment (Fig. 3A, compare lanes 4 and 7 with 5 and 8). Meanwhile, in monitoring the apoptotic marker PARP1, the results showed more cleavage to active forms of PARP1 in the KAP1 knockdown cells than that seen in control cells, as well as the Sin3A knockdown cells treated with TPA-NaB or hypoxia (Fig. 3A, compare lanes 5 and 8 with lanes 4 and 6 plus lanes 7 and 9). This further suggests that less KAP1 may result in more sensitivity to extracellular stresses during TPA-NaB and hypoxia treatment.

To determine if KAP1 knockdown in PEL cells causes a less apoptotic response than Sin3A or nonspecific knockdown, we determined the cell cycle profile of those knockdown PEL cells with or without hypoxia treatment. Consistently, the data in Fig. 3B showed that the sub-G1/G0 population of KAP1 knockdown cells after treatment (36.5% to 36.8%) presented a much lower response to hypoxia than that seen with Sin3A (38.5% to 51.1%) or nonspecific control (35.5% to 60.1%) knockdown cells. The combined evidence above showed that KAP1, collaborating with Sin3A, is critical for silencing replication of the KSHV genome and is a key sensor involved in both TPA-NaB- and hypoxia-mediated signaling pathways.

High concurrence of RBP-Jκ and HIF-1α binding sites within KSHV promoters associated with KAP1 and Sin3A. Previous studies have shown that the transcription factor RBP-Jκ is targeted by KSHV for regulation of LANA and RTA expression and is important for induction of the switch from latency to lytic replication upon treatment with TPA-NaB (37-39). Interestingly, inhibition of KAP1 enhances KSHV reactivation not only in hypoxia but also upon TPA-NaB treatment. Therefore, we looked at the DNA binding sites of RBP-Jκ, HIF-1α, KAP1, and Sin3A, along with RTA and LANA, across the KSHV genome in normoxia and hypoxia by chromatin immunoprecipitation (ChIP) assays. Strikingly, the results showed that the major HIF-1α binding sites across the whole KSHV genome (about 46%) showed a high degree of overlap with KAP1 and Sin3A in the presence of RBP-Jκ and LANA in normoxia (Fig. 4A and B). However, the association of KAP1 and Sin3A within the KSHV genome was significantly decreased in hypoxia (Fig. 4A and B). No dramatic change in the protein levels of both KAP1 and Sin3A were observed in normoxia and hypoxia in our previous study (26). Interestingly, analysis of the major HIF-1α binding sites under hypoxic stress identified four clusters, HRC I, II, III, and IV, with a relative higher degree of the conserved HRE (ACGTGC) sequence within these KSHV genomic regions (compare Fig. 4A with Fig. 5A, bottom). Further, there were several important latent and lytic genes, including those for RTA and LANA, located in HRC II and...
IV, respectively (Fig. 5A, top). To further confirm whether the viral genes within these four HRC regions respond to hypoxic stress, the transcriptional levels of each gene in PEL cells with 1% oxygen treatment at different time points (0, 6, 24, and 36 h) were determined by quantitative PCR. The TPA-NaB-treated cells were used as a parallel control. We did observe that most of the 15 genes were gradually enhanced with hypoxic treatment within a 36-h period (Fig. 5A, middle). Consistent with the results in Fig. 3A, we saw that the expression of LANA was greatly induced by hypoxia but not TPA-NaB within 36 h of treatment. In contrast, the transcription levels of RTA after induction were relatively higher (2.5-fold by hypoxia and 5.1-fold by TPA-NaB) than those of LANA (Fig. 5A, middle and bottom). Interestingly, immediate early and early viral genes (ORF50 and ORF74) were higher in response to TPA-NaB than hypoxia, while late viral genes (ORF22, ORF25, ORF29, and ORF44) in general showed an opposite response (Fig. 5A, middle and bottom). In addition, upon TPA-NaB induction, we also observed that the gene promoters of ORF50, ORF47, ORF16, and ORF59, containing both the RBP-J site oligonucleotide (HRE) association with the HRE-associated hypoxia-responsive genes is dependent on the transcriptional activity of RTA during hypoxia. By analyzing the ORF50 and ORF60 gene promoters, which individually contained both the HRE-RBS and HRE alone, the results of BC3 ChIP assays with or without hypoxia treatment with antibodies against KAP1, Sin3A, RBPs, HIF-1α, RTA, or LANA revealed that the concurrence of RBS with HRE at the same promoter not only enhanced KAP1 and Sin3A association with the HRE gene promoters in normoxia (Fig. 5B, compare lanes 5 and 7 with lanes 17 and 19) but also increased the association of RTA and RBPs-Jk in hypoxia 14-fold and 3.3-fold, respectively (Fig. 5B, compare lanes 15 and 21 with lanes 16 and 22). In contrast to the HRE promoter alone, the association of RTA and RBPs-Jk in hypoxia increased only 2.0-fold and 1-fold, respectively (Fig. 5B, compare lanes 3 and 9 with lanes 4 and 10).

Inhibition of KAP1 enhanced the association of RBPs-Jk with HIF-1α in both normoxia and hypoxia. To investigate whether RBPs-Jk is involved or is a component of the LANA-RBP-Jk-mediated HIF-1α complex, including KAP1 in hypoxia, we determined the levels of endogenous RBPs-Jk associated with a HIF-1α binding site oligonucleotide (HRE) in vitro in PEL cells with or without KAP1 knockdown under normoxic and hypoxic conditions. The results showed that the level of RBPs-Jk associated with HIF-1α was greatly enhanced in hypoxia, with a 4.5-fold increase for nonspecific knockdown control and a 6-fold increase for KAP1 knockdown (Fig. 6A). Strikingly, as shown in Fig. 6B, the results from luciferase reporter assays of the RTA promoter containing HRE and RBS (RBPs-Jk binding site) or HRE alone in the presence or absence of KAP1 suggested that in addition to enhancing RBPs-Jk-binding activity, the RBPs-Jk binding sites along with HRE further enhanced the transcriptional activity of the RTA promoter in hypoxia, from 2- to 6.2-fold in the knockdown control group (Fig. 6B, compare lanes 1 and 2 with lanes 5 and 6) and from 2.5- to 3.1-fold in the KAP1 knockdown group (Fig. 6B, compare lanes 3 and 4 with lanes 7 and 8). We also observed that the presence of RBS did reduce the transcriptional level of the RTA promoter in the nonspecific control instead of the KAP1 knockdown group under the normoxic condition (Fig. 6B, compare lanes 1 and 3 with lanes 5 and 7). These data support the hypothesis that the concurrence of RBS with HRE could be targeted by KSHV for selectively regulating specific gene promoters with HRE during normoxia and/or hypoxia.

To further analyze whether there is a significant difference in the concurrence rates of RBS and HREs within the same gene promoters in the host cellular and KSHV genome in order for the virus to selectively regulate cellular and viral genes, we statistically analyzed the conserved RBS and HRE sites of each gene promoter length within 500 bp across both the host cellular and KSHV genomes with a stringent P value cutoff. Intriguingly, the results showed that the concurrence rate of RBS and HRE was significantly higher than that seen in the human genome (46.8% versus 34.1% as shown in Fig. 6C; raw data in Table 2). This indicated that RBPs-Jk could be a second subtle regulator along with KAP1 which is targeted by KSHV to differentiate between its own and the host cellular gene promoters containing HRE in response to the accumulated HIF-1α in normoxia and hypoxia.

**DISCUSSION**

Increasing evidence suggests that KAP1, a member of the tripartite motif (TRIM) protein family which displays antiviral properties in addition to its documented transcriptional corepressor function, is also involved in various biological functions (including complex function with HDACs leading to reversible acetylation of nonhistone proteins [such as p53 and E2F1]) and mediates inhibition of viral integration (40). The different molecular mechanisms of KAP1 restriction parallel its dual activities at the cellular level: as a transcription regulator functioning as a corepressor and as a regulator of protein activity through acetylation. Hence, the absence of KAP1 in transcriptional repression will disfavor a hypothetical role for KAP1 in viral persistent infection. Consistent with the fact that KAP1 and Sin3A are chromatin silencers, our finding showing that inhibition of KAP1 or Sin3A in PEL cells led to high sensitivity to hypoxic stress in terms of viral reactivation further supports this hypothesis.

In regard to KSHV, it is well known that latency is the dominant form of KSHV persistent infection associated with host cell transformation. However, reactivation from latency is also impor-
tant for the growth and dissemination of virus-associated tumor cells (41). KSHV reactivation by TPA-NaB has been extensively documented by directly or indirectly targeting transcriptional activation of the promoter regulating the lytic master regulator RTA (ORF50) (42). However, it is not fully understood how hypoxia induces reactivation from KSHV latency. To address this question, we and other groups have found that hypoxia not only induces the key latent antigen LANA encoded by KSHV but also
induces lytic replication during hypoxia, we recently demonstrated that LANA can target the hypoxia-sensitive chromatin remodeler KAP1 through its SUMO2-interacting motif (LANA$^{SIM}$) (26). Deletion of LANA$^{SIM}$ motif not only disrupts maintenance of the KSHV episome but also turns on lytic gene expression (26). However, the detailed mechanism of KAP1 involvement in the hypoxic response still remains unclear. In this report, we show that KAP1 knockdown not only led to a dramatic loss of KSHV episome copies but also enhanced the efficiency of reactivation in response to hypoxia. Importantly, we also reveal that there are at least four hypoxia-responsive sites within the KSHV genome and that the concurrence of the DNA binding site of both RBP-J$^k$ and HIF-1$\alpha$ (which is the downstream transcriptional effector of TPA-NaB and hypoxia stimuli) within the same gene promoter is high and tightly associated with KAP1. Thus, our studies indicate that RBP-J is a second subtle regulator along with KAP1 which is targeted by KSHV for selectively silencing hypoxia-responsive viral and cellular gene expression in normoxia. RBP-J$^k$ coordinates with HIF-1$\alpha$ to initiate expression of HRE-containing lytic genes, including that for RTA in the absence of the inhibitory function of KAP1 during hypoxia. This production of RTA therefore functions as a third factor to further enhance its own expression and, directly or indirectly, that of other RTA-dependent genes (Fig. 7). RBP-J$^k$ is a key responder to TPA-NaB stimulation for KSHV lytic replication, and TPA-NaB-induced lytic protein vPK (ORF36) can block the repressive activity of KAP1 (14). Our discovery further supports the notion that KAP1 is a repressor for KSHV lytic reactivation in response not only to hypoxia but also to TPA-NaB. However, in contrast, hypoxia but not TPA-NaB treatment greatly enhanced the expression of LANA, which is consistent with the luciferase unit fold induction of pRta-Luc compared with pGL-3 vector alone.

FIG 6 The transcription activities of gene promoters containing HREs in hypoxia are impaired by both KAP1 and RBP-J$^k$. (A) Inhibition of KAP1 enhances the association of RBP-J$^k$ with HIF complex in hypoxia. In vitro DNA-binding assays of RBP-J$^k$ were performed under normoxic and hypoxic conditions. Nuclear cell extracts from BC3/shCtrl or BC3/shKAP1 with 21% or 1% oxygen treatment overnight were individually incubated with wild-type (Wt) or mutant (Mut) HIF-1$\alpha$-binding DNA oligonucleotide followed by three washes, and the precipitations were Western blotted with RBP-J$^k$ antibodies. The relative density (RD) of RBP-J$^k$ binding affinity is presented. (B) Inhibition of KAP1 dramatically enhances the transcriptional level of the RTA promoter with a RBP-J$^k$ binding site in hypoxia. 293 cells were transfected with the luciferase reporter of the RTA promoter (pRta-Luc) with RBS and HRE or HRE alone in the presence or absence of KAP1 knockdown (shKAP1). At 36 h posttransfection, cells were subjected to treatment with or without 1% oxygen for 12 h before harvest. The results are shown as the RLU (relative luciferase unit) fold induction of pRta-Luc compared with pGL-3 vector alone. Data are presented as means ± standard deviations of three independent experiments. (C) Percentages of HREs and RBP-J$^k$ binding sites (RBS) within the same gene promoter of total HREs in host cellular and KSHV genomes. The data are summarized from Table 2. The sequence logo of conserved RBS used for analysis is shown at the bottom.

cooperates with HIF-1$\alpha$ bound to HREs within the RTA promoter (10, 43) to directly induce lytic gene expression (i.e., ORF34-37) for lytic replication (44). Interestingly, we and other groups have found that HIF-1$\alpha$ is stabilized and accumulated in both PEL and KS cells in normoxia (29, 36). To further address how KSHV controls its own latent infection with HIF-1$\alpha$ accumulation while it induces lytic replication during hypoxia, we recently demonstrated that LANA can target the hypoxia-sensitive chromatin remodeler KAP1 through its SUMO2-interacting motif (LANA$^{SIM}$) (26). Deletion of LANA$^{SIM}$ motif not only disrupts maintenance of the KSHV episome but also turns on lytic gene expression (26). However, the detailed mechanism of KAP1 involvement in the hypoxic response still remains unclear. In this report, we show that KAP1 knockdown not only led to a dramatic loss of KSHV episome copies but also enhanced the efficiency of reactivation in response to hypoxia. Importantly, we also reveal that there are at least four hypoxia-responsive sites within the KSHV genome and that the concurrence of the DNA binding site of both RBP-J$^k$ and HIF-1$\alpha$ (which is the downstream transcriptional effector of TPA-NaB and hypoxia stimuli) within the same gene promoter is high and tightly associated with KAP1. Thus, our studies indicate that RBP-J$^k$ is a second subtle regulator along with KAP1 which is targeted by KSHV for selectively silencing hypoxia-responsive viral and cellular gene expression in normoxia. RBP-J$^k$ coordinates with HIF-1$\alpha$ to initiate expression of HRE-containing lytic genes, including that for RTA in the absence of the inhibitory function of KAP1 during hypoxia. This production of RTA therefore functions as a third factor to further enhance its own expression and, directly or indirectly, that of other RTA-dependent genes (Fig. 7). RBP-J$^k$ is a key responder to TPA-NaB stimulation for KSHV lytic replication, and TPA-NaB-induced lytic protein vPK (ORF36) can block the repressive activity of KAP1 (14). Our discovery further supports the notion that KAP1 is a repressor for KSHV lytic reactivation in response not only to hypoxia but also to TPA-NaB. However, in contrast, hypoxia but not TPA-NaB treatment greatly enhanced the expression of LANA, which is consistent with the luciferase unit fold induction of pRta-Luc compared with pGL-3 vector alone.

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TABLE 2 Analysis of HRE and RBS within the gene promoter from host cellular and KSHV (GenBank accession number NC_009333) genome sequence$^*$

<table>
<thead>
<tr>
<th>Genome parameter</th>
<th>No. of HREs (nt)</th>
<th>No. of genes with both RBS and HRE</th>
<th>No. of genes with RBS (ACGTGC)</th>
<th>No. of genes with RBS and HRE (ACGTGC)</th>
<th>No. of genes with HRE (ACGTGC)</th>
<th>No. of genes with HRE (both HRE)</th>
<th>No. of genes with both RBS and HRE and HRE (both HRE)</th>
<th>Total gene no.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host Cellular</td>
<td>27028</td>
<td>3,688</td>
<td>9,236</td>
<td>5,394</td>
<td>7</td>
<td>6</td>
<td>2,13E-06</td>
<td>3,587</td>
<td>1.70E-06</td>
</tr>
<tr>
<td>KSHV</td>
<td>43</td>
<td>6881</td>
<td>10,851</td>
<td>3,053</td>
<td>14</td>
<td>6</td>
<td>4,02E-05</td>
<td>10,851</td>
<td>1.70E-06</td>
</tr>
</tbody>
</table>

$^*$ Conservation P value is obtained by a permutation test. It means that the probability of getting a sequence with the conservation randomly is less than 0.05. TFBS scan P value shows the probability of getting a sequence with the same similarity between the detected TFBS sequence and those in the motif database randomly is less than 0.001. Concurrence P value is obtained by hypergeometric test. It indicates the significance of the concurrence between the genes with HRE and those with RBS. For cross-species conservation, the human host cellular genome is compared with that of the squirrel monkey, while KSHV is compared with saimirine herpesvirus 2.
tent with a recent report showing that hypoxia enhanced LANA expression (43). In addition, the role of KAP1 in response to extracellular stress like hypoxia or TPA-NaB treatment is not unique. Other groups and our current studies have shown that KAP1 responds to cells treated by serum starvation (data not shown) as well as DNA-damaging agents to arrest the cell cycle and apoptosis, respectively (45). These results indicate that KAP1 is a global transcriptional corepressor of KSHV lytic gene promoters through association with two key DNA-binding proteins, RBP-Jκ and HIF-1α, and can play a critical role in maintaining viral latency. A recent report showing that the KSHV-encoded latent protein kaposin B also deregulates KAP1-mediated inhibition of STAT3 further demonstrates an important role for KAP1 in KSHV latency infection (27).

How cellular signaling pathways cross talk is a rapidly emerging field in many branches of biology. The recent elucidation of the Notch-hypoxia intersection indicates the existence of cross talk between hypoxia and other key signaling (including Notch) mechanisms (46) and that interactions of two signaling pathways can occur at multiple steps in the signaling cascade (46). RBP-Jκ (also called CSL) is the major downstream transcription factor of the Notch signaling pathway and is a sequence-specific DNA binding factor which recruits corepressor or coactivator complexes to its cis-regulatory binding elements within the target gene promoters (47). It has recently been reported that RBP-Jκ deficiency triggers viral replication but attenuates reactivation by TPA-NaB for virion production in KSHV-infected B cells. This indicates that RBP-Jκ is required for both latency maintenance and lytic reactivation (48). In addition, RTA as the key master regulator of lytic replication has been demonstrated to either directly bind to DNA or use other DNA binding factors like RBP-Jκ as DNA adaptors. Consistent with previous reports (49–51), our studies also showed that there are a total of about 18 RTA binding sites within KSHV genome, which are tightly located in KSHV genes, including PAN RNAs, ORF16, ORF29, ORF45, ORF50, ORFK8, ORFK9, ORF71/72, ORFK14/ORF74, and ORFK15. Also, most of the viral transcripts that encode structural proteins are expressed following transcripts for the replication proteins. Intriguingly, a KSHV nucleotide sequence search with well-known HRE and RBS sequences from cellular genes found 58 individual matches for HIF-1α and 43 for RBP-Jκ within the KSHV genome. Many of them are located at regions containing known or speculated lytic gene promoters (52). For example, ORF57, ORF59, and ORF47 activation by RTA is dependent on RBP-Jκ binding (38, 53). Since RBP-Jκ appears to be a signaling molecule controlling cellular survival and the viral life cycle of KSHV-infected cells, it is not surprising that RBP-Jκ is identified as a critical molecule engaged in response to hypoxic stress. What we observed about collaboration of HIF-1α and RBP-Jκ in KSHV suggests a cross talk between HIFα and Notch signaling occurring at the intersection between the oxygen-sensing and developmental pathways. Notch has been reported to potentiate HIF-1α-mediated transactivation of hypoxia-inducible genes in the context of both tumorigenesis and cellular differentiation (46, 54). Conversely, HIF-1α has also shown to enhance Notch signaling through several distinct mechanisms (46, 54–56). HIF-1α has been shown to stabilize Notch ICD in both normoxia and hypoxia in a manner independent of its transcriptional activity (46, 54), although the precise mechanism remains unclear. Meanwhile, HIF-1α interaction with Notch at the HEY2 and HES1 promoters has also been reported, and this association augments Notch-mediated transactivation under hypoxia in a manner that depends upon a functional CTAD but not DNA binding of HIF-1α (55, 56). This supports our ob-

FIG 7 Schematic illustrating the role of both KAP1 and RBP-Jκ in hypoxia-induced KSHV reactivation. There are at least four hypoxia-responsive clusters (HRC I, II, III, and IV) within KSHV chromatin. During latent infection, to efficiently silence HRE-responsive gene expression and maintain the viral episome, LANA334-mediated chromatin silencer KAP1 targets the gene promoter of each HRC region with a high concurrence of RBP-Jκ binding site and HRE to inhibit viral gene expression (i.e., RTA) in normoxia. In contrast, each HRC region of KSHV chromatin in hypoxia (1% O2) undergoes three steps to reactivate lytic replication. In step 1, loss of SUMO-modified KAP1 induced by hypoxia (confirmed by shRNA-mediated inhibition of KAP1) remodels the chromatin structure and cellular survival and the viral life cycle of KSHV-infected cells, it is not surprising that RBP-Jκ is identified as a critical molecule engaged in response to hypoxic stress. What we observed about collaboration of HIF-1α and RBP-Jκ in KSHV suggests a cross talk between HIFα and Notch signaling occurring at the intersection between the oxygen-sensing and developmental pathways. Notch has been reported to potentiate HIF-1α-mediated transactivation of hypoxia-inducible genes in the context of both tumorigenesis and cellular differentiation (46, 54). Conversely, HIF-1α has also shown to enhance Notch signaling through several distinct mechanisms (46, 54–56). HIF-1α has been shown to stabilize Notch ICD in both normoxia and hypoxia in a manner independent of its transcriptional activity (46, 54), although the precise mechanism remains unclear. Meanwhile, HIF-1α interaction with Notch at the HEY2 and HES1 promoters has also been reported, and this association augments Notch-mediated transactivation under hypoxia in a manner that depends upon a functional CTAD but not DNA binding of HIF-1α (55, 56). This supports our ob-

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servation that RBP-Jk could bind to HIF-1α, and it requires further investigation.

In addition, to address the physiological relevance of KSHV to selectively activate gene expression via the virus-regulated HIF-1α, we have found that the transcription factor RBP-Jk is also associated with the LANA-associated inhibitory complex and responds to hypoxic stress. The fact that a higher percentage of RBP-Jk binding sites concurrently associates with HRE on the KSHV genome (particularly at the RTA promoter) than that seen on the human genome (i.e., VEGF promoter) may explain why KSHV can silence viral lytic gene expression and selectively activates cellular genes through accumulation of HIF-1α in normoxia. However, this accumulation strongly reactivates lytic replication in hypoxia.

In summary, our study provides new mechanistic insights on the programmed regulation of the KSHV genome during latent and lytic replication controlled by the major latent antigen LANA for gene silencing or activation based on KAP1. Particularly, the discovery that the LANA-associated KAP1 complex is highly sensitive to hypoxia (a physiologically relevant environmental condition) provides a potential therapeutic target against KSHV-associated cancers.

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