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Kaposi's Sarcoma-Associated Herpesvirus Latent Protein LANA Interacts with HIF-1α To Upregulate RTA Expression during Hypoxia: Latency Control under Low Oxygen Conditions

Qiliang Cai, Ke Lan, Subhash C. Verma, Huaxin Si, Doug Lin, and Erle S. Robertson*

Department of Microbiology and the Tumor Virology Program, Abramson Comprehensive Cancer Center, University of Pennsylvania Medical School, 201E Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, Pennsylvania

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Hypoxia can induce lytic replication of Kaposi's sarcoma-associated herpesvirus (KSHV) in primary effusion lymphoma (PEL) cells. However, the molecular mechanism of lytic reactivation of KSHV by hypoxia remains unclear. Here we show that the latency-associated nuclear antigen (LANA), which plays a crucial role in modulating viral and cellular gene expression, directly associated with a low oxygen responder, hypoxia-inducible factor-1α (HIF-1α). LANA enhanced not only the transcriptional activities of HIF-1α but also its mRNA level. Coimmunoprecipitation and immunofluorescence studies documented a physical interaction between LANA and HIF-1α in transiently transfected 293T cells as well as in PEL cell lines during hypoxia. Through sequence analysis, several putative hypoxia response elements (HRE-1 to -6) were identified in the essential lytic gene *Rta* promoter. Reporter assays showed that HRE-2 (−1130 to −1123) and HRE-5 and HRE-6 (+234 to +241 and +812 to +820, respectively, within the intron sequence) were necessary and sufficient for the LANA-mediated HIF-1α response. Electrophoretic mobility shift assays showed HIF-1α-dependent binding of a LANA protein complex specifically to the HRE-2, -5, and -6 motifs within the promoter regulatory sequences. This study demonstrates that hypoxia-induced KSHV lytic replication is mediated at least in part through cooperation of HIF-1α with LANA bound to the HRE motifs of the *Rta* promoter.

The ability of cells to adapt to periods of low oxygen (hypoxia) is important for their survival in both physiological and pathophysiological states (3). Regions of hypoxia are known to exist within many tumors, and the extent of tumor hypoxia correlates with prognosis for a number of tumor types (15, 34, 36). The cellular response to hypoxia involves diverse processes, including glycolysis and angiogenesis. This response is mediated by a transcriptional complex referred to as hypoxia-inducible factor 1 (HIF-1), which consists of α and β subunits.

HIF-1α is a basic helix-loop-helix transcription factor, and its expression is highly regulated in normoxic cells. Reduction in cellular oxygen concentration results in an exponential induction and accumulation of HIF-1α (39). Under hypoxic conditions, HIF-1α interacting with HIF-1β binds to hypoxia-responsive elements (HREs) within the downstream gene promoter. The core consensus sequences shown to be bound by the HIF-1 heterodimer have been identified as TCAGGTG (27), BACGTSSK (B = G/C/T, S = G/C, and K = G/T) (9, 21), and 5′-RCGTG-3′ (R = G/A) (33), although 5′-RCGTG-3′ recently has been used as the core sequence (33). The specific binding of HIF-1 to the HRE activates the transcription of more than 60 specific cellular genes, including vascular endothelial growth factor (VEGF) and glucose transporter-1 (Glut-1) (23). Hypoxia in the tumor microenvironment is sufficient to activate HIF-1-dependent gene expression of downstream responsive cellular genes (4). The induction of these cellular molecules results in increased oxygen availability by promoting erythropoiesis and angiogenesis (32), leading to a more aggressive tumor phenotype (14).

The Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, has been implicated as a major contributor to the pathogenesis of Kaposi's sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman's disease (8). Like other herpesvirus, KSHV exhibits two distinct phases in its life cycle, latent and lytic replication. During latent infection, a limited number of KSHV genes are expressed in the KSHV-infected cells. The product corresponding to one of these genes is referred to as the latency-associated nuclear antigen (LANA), which plays a crucial role in the replication and maintenance of the viral episomal genomes in KSHV-infected cells (18, 19, 30). In addition, LANA has also been shown to function as a transcriptional coactivator through its physical interaction with several cellular proteins, including the p53 (11), pRB (29), ATF4/CREB2 (24), Sp1 (37), c-Jun (1), and STAT3 (28) transcriptional factors.

Upon chemical induction, KSHV produces RNA transcripts from its immediate-early genes, which results in the expression of viral transcription activator proteins such as ORF50 and K8, which are important for induction of lytic replication (40). ORF50, a homolog of the Epstein-Barr virus immediate-early gene product RTA, is known to function as an essential transcriptional activator inducing the lytic cycle of KSHV and is expressed earlier than K8 (26, 35). RTA activates expression of the early and late genes in the KSHV lytic cycle (25). Recently, it has been demonstrated that hypoxia can also induce lytic replication of KSHV (6). Although it has been shown that the KSHV genome contains hypoxia-responsive elements (13), the

* Corresponding author. Mailing address: Department of Microbiology and the Tumor Virology Program, Abramson Comprehensive Cancer Center, University of Pennsylvania Medical School, 201E Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA. Phone: (215) 746-0114. Fax: (215) 898-9557. E-mail: erle@mail.med.upenn.edu.

molecular mechanism by which KSHV responds to hypoxia to switch between the latent and lytic life cycles is still unclear.

In the present study, we examined the physical and functional interactions between HIF-1α and LANA in KSHV-infected PEL cell lines. Moreover, we have identified six putative elements in the promoter region of the KSHV lytic gene *Rta* that mediate hypoxic responses, and we demonstrate that these elements form complexes with HIF-1α and LANA. Thus, these data show that HIF-1α cooperates with LANA to upregulate lytic *Rta* gene expression when KSHV-infected cells are exposed to hypoxia.

MATERIALS AND METHODS

Plasmid constructs, cell culture, and transfection. Plasmids pA3M/LANA (FL), pA3M/LANA1-435 (N), pA3M/LANA762-1162 (C), pA3M/LANA301-942 (M), pA3M/LANA1-327^929–1162 (ΔM), and pA3M were described previously (2, 37). pCEP4/HIF-1α was a gift from Gregg L. Semenza of Johns Hopkins University (10). Plasmid pH800, containing full-length HIF-1α promoter (bp −541 to bp 0) and luciferase reporter, was kindly provided by Emmanuel Minet of Facultés Universitaires de la Paix (27). Luciferase reporter plasmid pRpluc1-3087 with an *Rta* promoter (~3 kb upstream) was a kind gift from Ren Sun of the University of California, Los Angeles (7). A series of truncation plasmids of pRpluc was made to remove HREs within the *Rta* upstream regulatory sequence (see Fig. 6B). The truncated promoters were named pRpluc1-2039, pRpluc1-1327, pRpluc1-941, pRpluc1-550, pRpluc1115-1327, pRpluc1679-2039, pRpluc550-941, pRplucΔ941-1679, and pRplucΔ209-1115. The constructs pRpluc1-2039 (contains HRE-1, HRE-2, HRE-3, and HRE-4), pRpluc1-1327 (deleted for the HRE-1 site), and pRpluc1-550 (in which HRE-1, HRE-2, and HRE-3 are deleted) were described in previous studies (22). To construct pRpluc1-941, in which HRE-1 and HRE-2 are deleted, pRpluc1-3087 was digested by NcoI and the large fragment was self-ligated. Similarly, pRplucΔ941-1679 (only HRE-2 deleted) and pRplucΔ209-1115 (both HRE-1 and HRE-3 deleted) were generated individually from pRpluc1-1327 with KpnI digestion, and pRpluc1-2039 was generated with NcoI digestion. To construct pRpluc1115-3127, containing only HRE-2, the large fragment of pRplucΔ209-1115 digested by KpnI and BglII was filled in by Klenow to blunt ends and ligated. Similarly, the pRpluc1679-2039 (containing only HRE-1) and pRpluc550-941 (containing only HRE-3) plasmids were generated individually from NcoI/BglII-digested pRplucΔ941-1679 and from KpnI/BglII-digested pRpluc1-941 with Klenow blunt ending and self-ligation. The splicing sequence (S1-976) of the *Rta* promoter, stretching from position 71596 to position 72572 in the KSHV genome, was obtained by PCR amplification with an upstream primer (BamHI, 5′-CGGGATCCATGGCGCAAGATGACAAGG-3′ [underlining indicates digested sites) and a downstream primer (BglII, 5′-GAAGATCTAATCCGAATGCACACATCTTC-3′) from GB21 cosmids (a kind gift from Yuan Yan of the University of Pennsylvania) and cloned individually into BglII-digested pGL-2 basic vector and pRpluc1-3087 to form plasmids pRplucS1-976 and pRpluc1-3087-S. The large-fragment self-ligation of pRplucS1-976 digested by HindIII and pRpluc1-3087-S digested by PstI generated plasmids pRplucΔS43-707 (containing only HRE-5) and pRplucS1-442 (containing only HRE-6), respectively.

Human embryonic kidney (HEK) 293 and 293T cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, penicillin, and streptomycin. The KSHV-negative cell line BJAB and the KSHV-positive cell lines BCBL-1 and BC-3 were maintained in RPMI 1640 medium with 7% fetal bovine serum, 4 μM L-glutamine, penicillin, and streptomycin. All cultures were incubated at 37°C in a humidified environment supplemented with 5% CO_2. Ten million cells in 400 μl medium were transfected by electroporation with a Bio-Rad gene pulser in 0.4-cm-gap cuvettes at 220 V and 975 microfarads. To establish a hypoxic condition, cells were allowed to grow for 12 h in the presence of 100 μM cobalt chloride ($CoCl_2$) before use and maintained during the experiments (31).

IP and Western blotting. After 48 h of incubation, the transfected cells were lysed in cold radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin [1 μg/ml], and pepstatin [1 μg/ml]) on ice and homogenized. The lysates were precleared with protein A/G-Sepharose beads (Amersham Biosciences, Piscataway, N.J.), and then incubated first with antibodies against HIF-1α or myc (1 μg) in immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM $MgCl_2$, 0.1% Tween 20, 0.1 M KCl, 1× protease inhibitor cocktail [Amersham Biosciences, Piscataway, NJ], and 0.5 mM dithiothreitol) overnight at 4°C with constant rotation and then with protein A/G (50/50)-Sepharose beads for 1 h. Beads were washed three times with IP buffer and resuspended in 40 μl of 2× sodium dodecyl sulfate Laemmli buffer. The sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analyses were performed using primary monoclonal antibodies against HIF-1α (BD Transduction Laboratory, San Jose, CA), myc (9E10), and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Detection and quantification were done using a Li-Cor Odyssey scanner (Li-Cor, Inc., Lincoln, NE).

Immunofluorescence. Cells transfected on coverslips were fixed in 3% paraformaldehyde for 20 min at room temperature. Antibody working dilutions were as follows: mouse anti-HIF-1α and rabbit anti-LANA at 1:50. Cells were permeabilized in immunofluorescence buffer (0.2% fish skin gelatin, 0.2% Triton X-100 in phosphate-buffered saline [PBS]) for 5 min and subsequently incubated for 1 h in immunofluorescence buffer with primary antibodies. Cells were washed three times in PBS. Fluorescently labeled Alexa Fluors, anti-rabbit 594 and anti-mouse 488 (Molecular Probes, Eugene, OR), were used as secondary antibodies for 30 min. Coverslips were washed in PBS and mounted on glass slides using Prolong antifade mounting medium (Molecular Probes, Eugene, OR) with 0.5 μM DAPI (4′,6′-diamidino-2-phenylindole) for nuclear staining (Pierce, Rockford, IL). All steps were performed at room temperature. Fluorescence confocal microscopy was performed with an Olympus microscope with FluoView FV300 software (Olympus, Melville, NY).

Real-time PCR. Quantitative real-time PCR was used to make a relative quantitative comparison of the mRNA levels of HIF-1α at different expression levels of LANA. Cells transfected with different doses of LANA were harvested at 48 h posttransfection. Total RNA was extracted using TRIzol reagent (Invitrogen, Inc.) following the manufacturer's instructions. cDNA was synthesized using a Superscript II reverse transcriptase kit (Invitrogen, Inc.), following the manufacturer's instructions. The primers used for real-time PCR were as follows: for HIF-1α, 5′-CCAGCAGACTCAAATACAAGAACC-3′ and 5′-TGTATGT GGGTAGGAGATG GAGAT-3′ (5), and for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-TGCACCACCAACTGCTTAG-3′ and 5′-GATGCA GGGATGATGTTC-3′. The cDNA was amplified using 10 μl of the DyNAmo SYBR green quantitative real-time PCR kit (MJ Research, Inc.), 1 mM each primer, and 2 μl of the cDNA product in a total volume of 20 μl. Thirty cycles, each of which were 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, were followed by 10 min at 72°C using an MJ Research Opticon II thermocycler (MJ Research, Inc.). Each cycle was followed by two plate readings, with the first at 72°C and the second at 85°C. A melting curve analysis was performed to verify the specificities of the amplified products. The values for the relative quantitation were calculated by use of the cycle threshold values for each sample tested in triplicate.

EMSA. Sequences of probes used for the electrophoretic mobility shift assay (EMSA) encompassing the putative HRE motifs and mutant probes are shown in Table 1. The ^{32}P-labeled probes were synthesized via the Klenow fill-in reaction and purified with Select-D G-25 columns (Shelton/IBI, Inc., Peosta, IA). Radioactive probes were diluted in water to a final concentration of 80,000 cpm/μl. DNA binding reactions were performed in a manner similar to that described previously (20). Proteins from nuclear extracts were mixed with 1 μg of poly(dC/dI) (Sigma-Aldrich, Inc., St. Louis, MO) in 1× DNA binding buffer for 5 min at room temperature; 1 μl of labeled probe was added to each reaction mixture, and the tubes were incubated at room temperature for 15 min. DNA-protein complexes were resolved in a nondenaturing 5% polyacrylamide gel electrophoresis. The gel was run in 0.5× Tris-borate-EDTA buffer at a constant voltage of 120 V for 4 h. Following electrophoresis, the gel was transferred to Whatman paper and dried for 1 h at 80°C. Dried gels were exposed to a phosphorimager screen for 12 h (Amersham Biosciences, Inc., Piscataway, NJ) and scanned by a PhosphorImager (Molecular Dynamics, Piscataway, NJ). For specificity determinations, cold competitor probe was added at a 200-fold molar excess. For supershift experiments, polyclonal antibodies against LANA and HIF-1α were added after an initial 30-min incubation of probe with nuclear extract and incubated for 1 h at 4°C.

Luciferase reporter assay. Ten million HEK 293 (or BJAB) cells transfected with luciferase reporter (5 μg) and different combinations of plasmids (the total amount of transfected DNA was adjusted with the appropriate blank vector) were harvested at 48 h posttransfection and subsequently washed once with PBS (Invitrogen, Inc.), followed by lysis with 200 μl of reporter lysis buffer (Promega, Inc.). A 40-μl portion of total lysate protein was mixed with 25 μl of luciferase assay reagent. Luminescence was measured for 10 s by use of an Optocomp I luminometer (MGM Instruments, Inc.). The luciferase activity was normalized with cotransfected β-galactosidase activity. Relative luciferase activity was expressed as activation (*n*-fold) relative to that of the reporter construct alone. The results shown represent experiments performed in triplicate.

TABLE 1. Oligonucleotides used for electrophoretic mobility shift assay

Oligonucleotide	Sequence[a]	Position[b]
wt1	5′-TACCACAAACAACC**TCAGGT**TTTCCTGCGACGC-3′	69602–69634
mut1	5′-TACCACAAACAACC**TTTAAT**TTTCCTGCGACGC-3′	
wt2	5′-GTATTCTGGATTCCAA**ACGTGC**CCAGCGGTACCCAA-3′	70450–70485
mut2	5′-GTATTCTGGATTCCAA**TTAATC**CCAGCGGTACCCAA-3′	
wt3	5′-ACTCCAACCGTC**TCACGT**CCGAGGTAATGTGCTC-3′	70707–70740
mut3	5′-ACTCCAACCGTC**TCTTAAC**CGAGGTAATGTGCTC-3′	
wt4	5′-GTAGCTGGGTGGCAATG**ACACGTC**CCCCTTTAAAAAGTC-3′	71502–71539
mut4	5′-GTAGCTGGGTGGCAATG**ACTTAAC**CCCCTTTAAAAAGTC-3′	
wt5	5′-GGAGAGTTAGG**GACGTGCT**GATTATGTGGAC-3′	71819–71849
mut5	5′-GGAGAGTTAGG**GTTTAACT**GATTATGTGGAC-3′	
wt6	5′-GTTTAGAGCGGG**TACGTGGC**AGTCTGGATTGA-3′	72396–72427
mut6	5′-GTTTAGAGCGGG**TTTAAGC**AGTCTGGATTGA-3′	

[a] Only the sequences of the sense oligonucleotides are shown. For the electrophoretic mobility shift assay, the complementary probes were used. The putative hypoxia response elements are shown in boldface type, and the mutations are underlined.
[b] The positions are relative to the KSHV genome.

Statistical analysis. Statistical analysis was performed by use of the Student *t* test; *P* values of <0.05 were considered statistically significant.

RESULTS

HIF-1α and LANA associate in human B cells and epithelial cells. Previous studies have shown that hypoxia is able to reactivate KSHV from latency (6). This fact led us to examine the molecular interactions between a key hypoxia responder, HIF-1α, and an abundant latent protein, LANA, in KSHV-infected cells. To assess whether LANA associates with HIF-1α, we first demonstrated the interaction between LANA and HIF-1α in cells by using coimmunoprecipitation assays for 293T cells that were transiently transfected with a LANA-myc expression construct under normoxic or hypoxic conditions. Whole-cell extracts of the transfected 293T cells were precipitated with anti-myc antibody, and the precipitates were analyzed by Western blotting with anti-HIF-1α and anti-myc antibodies. As shown in Fig. 1A, endogenous HIF-1α associated with LANA in cells. Importantly, LANA also associated with HIF-1α when the reverse immunoprecipitation with anti-HIF-1α antibody and subsequent Western blotting with anti-myc antibody were performed (Fig. 1A).

To determine if the association of HIF-1α and LANA occurred in the same compartments in the nucleus during hypoxia, we performed immunofluorescence assays on the KSHV-positive BCBL-1 PEL cell line. After 24 h of chemical hypoxia treatment, the BCBL-1 cells were harvested, fixed, and probed with mouse monoclonal antibody against HIF-1α and a human antiserum to test for specificity to LANA. Fluorescently labeled Alexa Fluors conjugated to the appropriate secondary antibodies were used for visualization of the proteins. The results showed that HIF-1α and LANA colocalized to a large extent in the same nuclear compartments of BCBL-1 cells during hypoxia (Fig. 1B).

To delineate the domains in LANA that mediate the protein-protein interactions between LANA and HIF-1α, coimmunoprecipitation experiments were performed with a series of truncated LANA polypeptides (Fig. 1C, right panel). As shown in Fig. 1C, left panel, a construct with a deletion of the LANA central repeat domain (ΔM; deletion of amino acids [aa] 327 to 929 [Δ27-929]) and the N-terminal domain of LANA (N; aa 1 to 435) interacted with HIF-1α, whereas the C-terminal domain of LANA (C; aa 762 to 1162) failed to interact with HIF-1α (Fig. 1C). These data suggest that the N-terminal domain is required for interaction of LANA with HIF-1α.

We also detected the effect of the transient expression of LANA on endogenous HIF-1α levels in KSHV-negative B-lymphoma BJAB and HEK 293T cell lines. Consistent with the association data presented above, in both BJAB and 293T cell lines, the transient expression of LANA increased endogenous levels of HIF-1α in a dose-dependent manner (Fig. 2). These results suggest that LANA can induce levels of HIF-1α transcripts in KSHV-infected cells and is likely to regulate its expression through association with other transcription factors, including HIF-1α, as well as affecting the posttranslational stability of the protein.

LANA enhances the transcription level of HIF-1α mRNA. Previous studies have reported that LANA can associate with *cis*-acting elements and likely complex with other transcription factors to regulate gene expression (12, 16). Therefore, to investigate whether the elevation of HIF-1α protein levels was due to an increase in gene transcription, we determined the levels of HIF-1α transcripts by use of quantitative real time-PCR as well as reporter assays with an HIF-1α promoter fusion with luciferase reporter construct in LANA-expressing 293T cells. The real-time PCR analysis showed that LANA enhanced the relative levels of HIF-1α transcripts in the endogenous pool (Fig. 3A). The luciferase reporter assays further showed that LANA can indeed enhance transcription from the endogenous HIF-1α promoter by approximately 20-fold over that of the vector-alone control in 293T cells in a dose-dependent fashion (Fig. 3B).

To determine whether HIF-1α synergizes with LANA to affect the transcription of its own promoter, the luciferase activity generated from the HIF-1α promoter was assayed for 293T cells with LANA coexpressed with HIF-1α. The data showed that HIF-1α in the presence of LANA further induced HIF-1α transcription from its native promoter (Fig. 3B). Additionally, the reporter assays of the HIF-1α promoter with different truncations of LANA demonstrated that the amino domain of LANA was important for HIF-1α promoter activation (Fig. 3C).

HIF-1α cooperated with LANA to induce KSHV RTA expression during hypoxia. To explore the effect of HIF-1α on

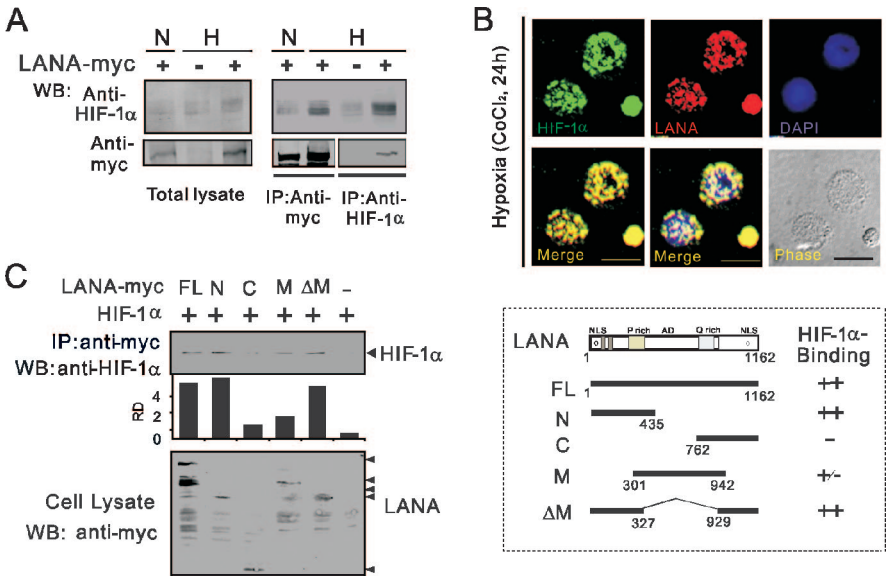

FIG. 1. HIF-1α and the KSHV latent protein LANA associate in vivo. (A) LANA coimmunoprecipitates with HIF-1α. HEK 293T cells were transfected with or without expression plasmid encoding myc-tagged LANA during normoxia (N) or 100 μM cobalt chloride-induced hypoxia (H). Forty-eight hours posttransfection, whole-cell extracts were prepared and immunoprecipitated (IP) using anti-myc (9E10) or anti-HIF-1α monoclonal antibody (BD Transduction Laboratory) and subsequently Western blotted (WB) with anti-HIF-1α or anti-myc antibodies (right panels). Total cell lysate (1%) was blotted with anti-myc or anti-HIF-1α antibodies (left panels). The results showed that LANA associates with endogenous HIF-1α. (B) Immunofluorescence assay for HIF-1α and LANA colocalization. After 24 h of chemical hypoxia treatment, KSHV-positive BCBL-1 cells were fixed with 3% paraformaldehyde and stained with human anti-KSHV serum (red) and mouse anti-HIF-1α (green). Micrographs are shown at magnifications of ×1,000 (bars, 20 μm) with DAPI nuclear staining (blue) and phase (gray). (C) Western blot analysis showing the amino-terminal domain of LANA specifically interacting with HIF-1α. Plasmids expressing amino 1- to 435-aa (N), carboxyl 762- to 1162-aa (labeled "C"), central 301- to 942-aa (M), and central deletion 327Ŷ29 (between aa 1 and 1162) (ΔM) truncations, as well as full-length LANA (aa 1 to 1162) (FL) with a myc tag, were cotransfected individually with pCEP4/HIF-1α in 293T cells. Forty-eight hours posttransfection, cell lysates were subjected to immunoprecipitation using anti-myc antibody and to Western blotting with anti-HIF-1α antibody (left panel). Total cell lysate (1%) was blotted with anti-myc to monitor the expression of LANA. The schematic on the right shows the domain of LANA binding with HIF-1α. RD, relative density.

KSHV infection, we determined the percentages of KSHV-infected cells positive for the lytic protein RTA at different times after hypoxia treatment. Ten million KSHV-positive BCBL-1 cells were cultured for 0, 12, 24, 48, and 72 h, during which times they were treated with $CoCl_2$ to induce a hypoxic environment. This was followed by immunofluorescence assays against HIF-1α and RTA proteins. As shown in Fig. 4, hypoxia rapidly induces HIF-1α and RTA expression in BCBL-1 cells, leading to a significant increase by 12 h of treatment. This increase reached a maximum level of about 40% induction in response to 48 h of treatment (Fig. 4). However, the most dramatic change was observed between 12 and 24 h of treatment (Fig. 4). Interestingly, a decrease in HIF-1α and RTA expression levels was observed by 72 h of treatment. These results suggest that KSHV-positive cells are induced to express RTA protein during exposure to hypoxia but that there is likely to be an inhibition of cell growth and that induction apoptosis may occur if cells are exposed to hypoxia for extended periods, i.e., longer than 48 h.

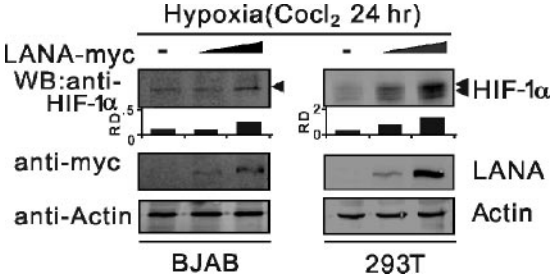

FIG. 2. The KSHV-encoded nuclear protein LANA regulates HIF-1α expression in a dose-dependent manner. Ten million hypoxia-treated Burkitt lymphoma BJAB cells and HEK 293T cells were transfected with different doses of plasmid pA3M/LANA normalized by the pA3M vector and maintained under hypoxic conditions. After 24 h of transfection, the cells were harvested and lysed for Western blot (WB) analysis using antibodies against HIF-1α, myc, or β-actin. The data show that HIF-1α expression was increased by LANA in a dose-dependent manner. RD, relative density.

To understand the physiological basis of the molecular interaction between HIF-1α and LANA, we further examined the effects of LANA on the activity of HIF-1α in KSHV-infected PEL cells. We transfected LANA-myc-expressed vector into hypoxic BCBL-1 or BC-3 cells and assessed HIF-1α and RTA protein levels. As shown in Fig. 5, in two KSHV-positive cell lines, we observed that overexpression of LANA further induced HIF-1α expression during hypoxia and, together with increased HIF-1α, can induce RTA expression (Fig. 5, lanes 2, 4, 6, and 8). These data indicate that in KSHV-infected PEL cells, HIF-1α cooperated with LANA to upregulate RTA levels during hypoxia.

Identification of the functional HREs in the KSHV *Rta* promoter. Typically, HIF-1α as a transcriptional factor can func-

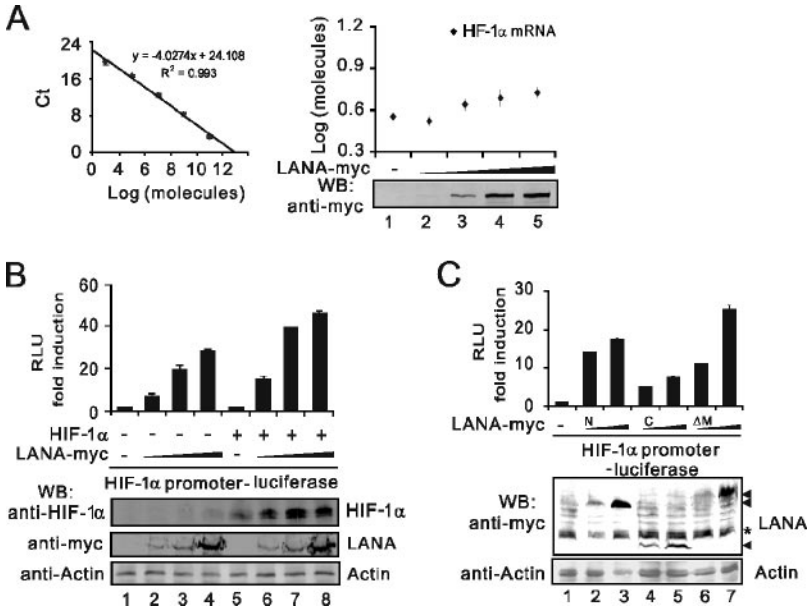

FIG. 3. The transcriptional activity of HIF-1α promoter is enhanced by LANA. (A) Quantitative real-time PCR analysis of HIF-1α transcripts in 293T cells transfected with different doses of LANA. After 48 h of transfection, total RANA was isolated from cells transfected with LANA. Real-time PCR was performed as described in Materials and Methods. HIF-1α transcript abundance was calculated by use of the cycle threshold (Ct) values, with the standard curve to the top. All samples and standards were tested in triplicate for the calculation of the mean and standard deviation. The data showed an increase in mRNA level of HIF-1α transcripts by LANA. The LANA expression was shown by Western blotting (WB) using anti-myc antibody. (B) LANA activates the transcription of the HIF-1α promoter. A fixed amount (5 μg) of full-length HIF-1α promoter-driving luciferase was cotransfected with different doses of pA3M/LANA (0, 1, 2.5, 5, or 10 μg) in the presence or absence of pCEP4/HIF-1α (total DNA amount was normalized by the vector alone) into 293T cells. The data show that the transcription levels of the HIF-1α promoter were increased by LANA and further induced by exogenous HIF-1α synergized with LANA. The numbers presented represent the relative light unit (RLU) change (*n*-fold) from the level for the reporter construct alone. Means and standard deviations from three independent experiments are shown. The levels of LANA and HIF-1α expression were shown by Western blotting using anti-myc and anti-HIF-1α with β-actin as a control. (C) The amino domain of LANA is required for HIF-1α promoter activation. Plasmids (5 or 10 μg) expressing the myc tag with amino 1- to 435-aa (N), carboxyl 762- to 1162-aa (C), and central deletion 327929 (between aa 1 and 1162) (ΔM) truncations of LANA were individually transfected with full-length HIF-1α promoter-driving luciferase (5 μg) into 293T cells. The luciferase activities were detected as described previously. The Western blot analysis against myc showed the expression levels of the LANA mutant forms. *, nonspecific band.

tion through interaction with specific *cis*-acting DNA binding sites, referred to as HREs, which are located within the promoter regulatory sequences of its target genes (9, 21, 33). In this study, sequence analysis of the KSHV *Rta* promoter revealed six putative HRE motifs showing homology with the HIF-1-binding consensus sequences TCAGGTG (27), BACG TSSK (9, 21), and 5′-RCGTG-3′ (33). The first potential HIF-1α-binding site, HRE-1, at positions −1981 to −1974 (positions 69615 to 69623 relative to the KSHV genome), shared homology with TCAGGTG in six of seven bases. The second site, HRE-2 (positions −1130 to −1123), shared homology with the BACGTSSK sequence in six of eight bases. The fourth site, HRE-4 (positions −77 to −70), and the sixth site, HRE-6 (+812 to +820, which lies within the intron sequence), shared homology with BACGTSSK in seven of eight bases, whereas the third site, HRE-3 (positions −877 to −870), and the fifth site, HRE-5 (intron sequence +234 to +241), shared homology with this sequence in all eight bases (Fig. 6A). However, corresponding to the RCGTG consensus sequence, only HRE-2, HRE-5, and HRE-6 contain complete conserved sequences (Fig. 6A). Thus, alignment with three HIF-1-binding motifs provides the critical consensus sequence represented as ASGT within the *Rta* promoter.

To determine which putative HREs are critical for HIF-1α to activate the *Rta* promoter, a series of truncated constructs of the *Rta* promoter fused to the luciferase reporter was generated. The HIF-1α-binding HRE sites were removed sequentially to determine the contribution of each site to the regulation of *Rta* expression (Fig. 6B, left). Transfection experiments in HEK 293 cells with these reporter constructs with or without pCEP4/HIF-1α, a HIF-1α expression vector, revealed that the elements HRE-2, HRE-5, and HRE-6 were important for HIF-1α-dependent activation (Fig. 6B, right). Deletion of the HRE-3 motif did not affect the luciferase activity. When the HRE-1 motif was deleted, the basal levels of luciferase activity were moderately decreased. However, this decrease did not significantly affect the HIF-1α-dependent activation of *Rta* promoter activity (Fig. 6B). Transfection experiments in which the truncated constructs contained only one putative HRE confirmed that the HRE-2, HRE-5, and HRE-6 motifs were all sufficient to mediate the HIF-1α-dependent activation of the *Rta* promoter response. The construct pRplucΔ209-1115, containing HRE-2 and HRE-4, showed a response to HIF-1α activation similar to that of the wild type, but the construct pRplucΔ941-1679, with the HRE-2 removed, did not. This confirms that the HRE-2 motif has a significant functional contribution to the activation of the *Rta* promoter (Fig. 6B). Similarly, within the *Rta* intron sequence, we also found that the

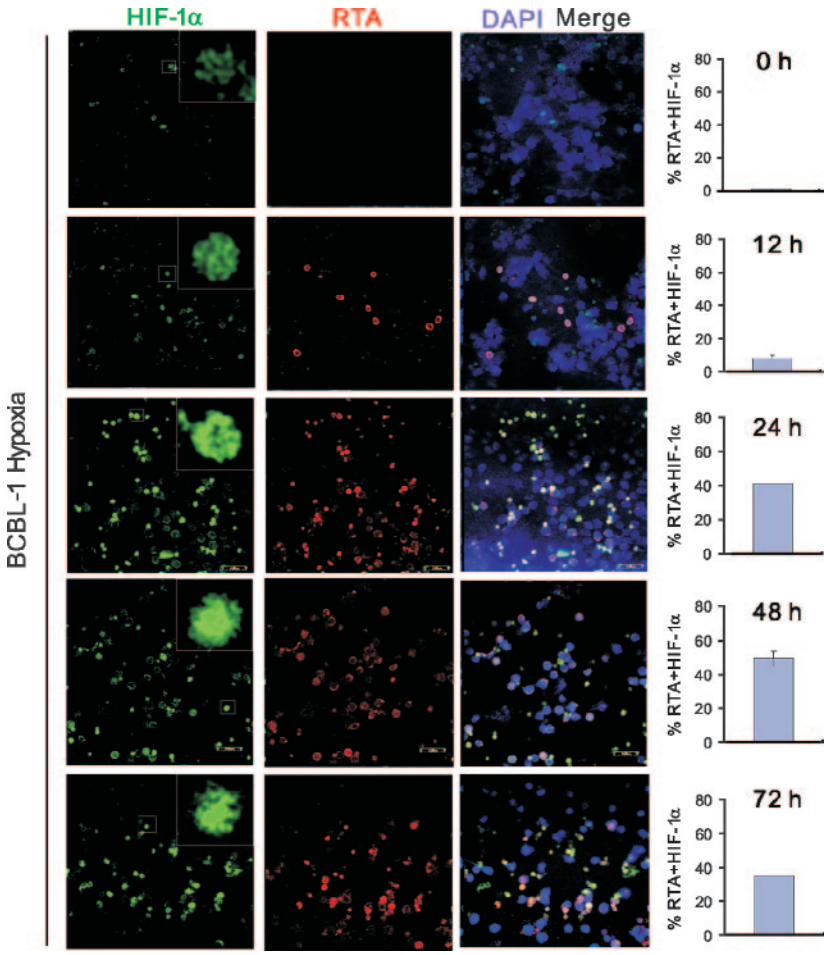

FIG. 4. Hypoxia induces RTA expression in cells latently infected with KSHV. KSHV-positive BCBL-1 cells were treated with 100 μM cobalt chloride for 0, 12, 24, 48, and 72 h and then subjected to immunofluorescence assays as described previously with mouse anti-HIF-1α monoclonal (BD Transduction Laboratory) and rabbit anti-KSHV RTA polyclonal (provided by Gary S. Hayward from Johns Hopkins University School of Medicine) antibodies. Fluorescently labeled Alexa Fluors goat anti-mouse 488 (green) and anti-rabbit 594 (red) (Molecular Probes, Eugene, Oreg.) were used as secondary antibodies. The nucleus (blue) was shown with DAPI (Pierce, Rockford, Ill.) staining. All micrographs are shown at magnifications of ×400 (bars, 50 μm). The percentages of both RTA- and HIF-1α-positive cells after hypoxia exposure are indicated in the right panels.

HRE-5 and HRE-6 motifs are both sufficient to mediate relatively high levels of HIF-1α-dependent activation (Fig. 6B).

The functional HREs within the *Rta* promoter are critical for the cooperation of HIF-1α with LANA in upregulating RTA expression under hypoxic conditions. Previous studies have shown that LANA does not bind directly to DNA but rather is indirectly targeted to the *Rta* promoter through interaction with the cellular DNA binding protein RBP-Jκ, the major downstream effector of the Notch signaling pathway (12, 16, 22). To determine whether LANA also affects the transcription of *Rta* through interaction with HIF-1α, as well as whether or not the functional HIF-1α-binding site plays any role in activating transcription of *Rta* by LANA during hypoxia, we used the deletion mutants of LANA in which the HIF-1α-binding domains were mutated and the wild type. The wild-type and mutant forms of LANA were transfected with or without HIF-1α in the presence of the reporter vector pRpluc1-3087, including the intron into HEK 293 and BJAB cells, and tested for luciferase activity in cell lysates. The results of the luciferase assays demonstrated that the presence of the LANA mutant with the carboxyl-terminal domain has little or no effect on the upregulation of the *Rta* promoter. However, dramatic activation was seen with the wild-type LANA and the amino-terminal domain (Fig. 7A). Furthermore, the transcriptional levels of the *Rta* promoter activated by endogenous HIF-1α and wild-type LANA seen for hypoxic conditions were higher than those seen for normoxic conditions, suggesting that LANA and HIF-1α interact cooperatively to upregulate the *Rta* promoter.

To address the roles of functional HREs within the *Rta* promoter on HIF-1α cooperation with LANA, a series of truncated *Rta* promoter constructs with HIF-1α-binding sites sequentially removed was used to determine the contribution of each site in the regulation of *Rta* expression by LANA and HIF-1α. These reporter constructs were transfected into HEK 293 cells along with the expression vector pA3M/LANA, pCEP4/HIF-1α, or both. As expected, significant upregulation of *Rta* promoter activity was observed when reporter con-

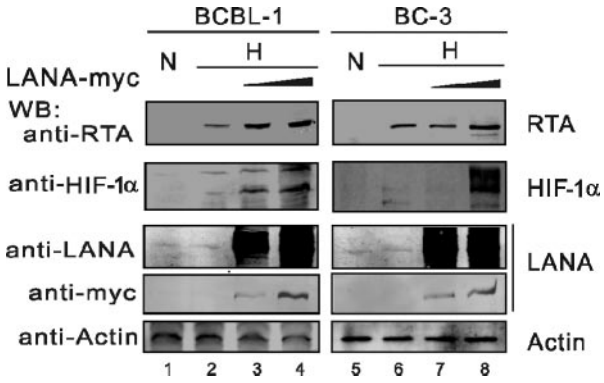

FIG. 5. LANA cooperates with HIF-1α to upregulate RTA expression during hypoxia. Ten million PEL BCBL-1 or BC-3 cells transfected with different doses of pA3M/LANA normalized by the pA3M vector were treated with 100 μM $CoCl_2$ for 24 h. The 40-μg portions of total protein from transfected cell lysates were analyzed by Western blotting (WB) using mouse anti-RTA (provided by Koichi Yamanishi of Osaka University, Osaka, Japan), anti-HIF-1α (BD Transduction Laboratory), anti-myc (for LANA), or anti-β-actin (Cell Signaling) antibodies. The data showed that RTA expression in PEL cells along with HIF-1α expression was increased more during hypoxia (H) than during normoxia (N) and was further induced by coexpression of LANA.

structs containing the *Rta* promoter with the HIF-1α-binding site HRE-2 were cotransfected with the LANA and HIF-1α expression vectors (Fig. 7B). This activation was not observed when cotransfections were performed with reporter constructs with the HRE-2 element deleted (Fig. 7B). Similarly, the elements HRE-5 and HRE-6 within the intron sequence of *Rta* are also critical for HIF-1α cooperation with LANA to upregulate the *Rta* promoter (Fig. 7B).

LANA interacts with HIF-1α bound to its cognate sequences within the *Rta* promoter in vitro. The results of the luciferase reporter assays describe above indicate that the potential HIF-1α-binding sites HRE-2, HRE-5, and HRE-6 within the *Rta* promoter are critical for LANA's ability to activate the promoter. Therefore, to test whether LANA activated the *Rta* promoter through the formation of a HIF-1α/LANA complex bound to the *cis*-acting DNA element, electrophoretic mobility shift assays were performed. The wild-type and mutant probes for EMSAs were designed to individually consist of the six potential HRE elements from the *Rta* promoter (Table 1). Double-stranded DNA probes for the HIF-1α-binding site were labeled and tested for binding to HIF-1α protein from

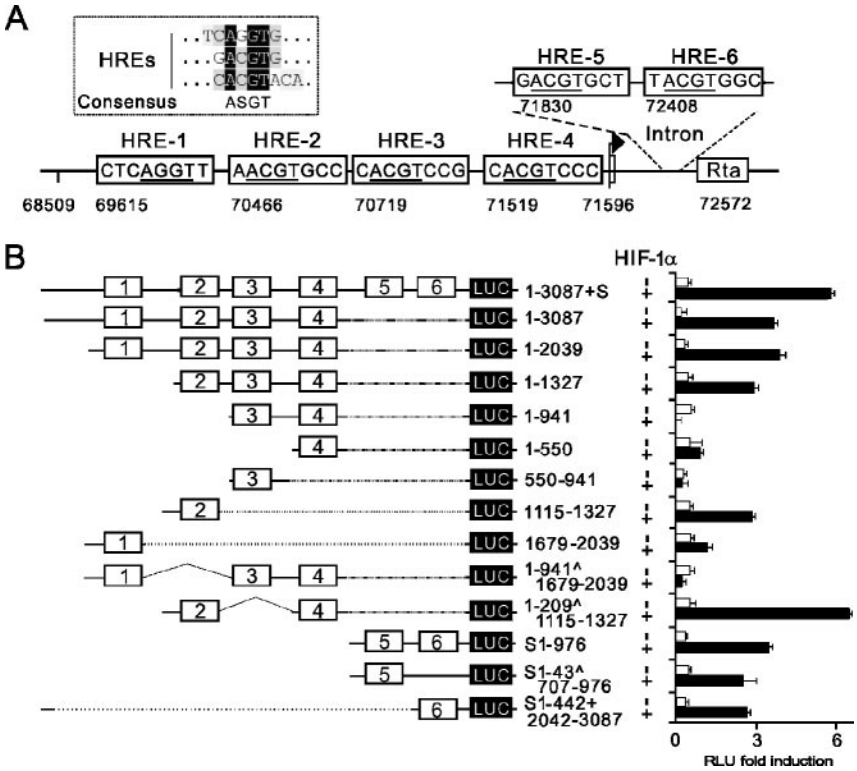

FIG. 6. The KSHV *Rta* promoter contains functional hypoxia-responsive elements. (A) Prediction of hypoxia-responsive elements in KSHV *Rta* promoter regions. The promoter region of KSHV lytic gene *Rta*, stretching from coordinates 68509 to 72572 relative to the KSHV genome, was aligned with the homologous sequence from conserved HREs (shown in dotted box). The six putative HREs (HRE-5 and -6 are located in the splicing sequence from 71596 to 72572) are highlighted in the box, and underlined letters indicate homology with the canonical HRE sequence ASGT from previous studies (9, 27, 33). (B) Transcriptional activation of the KSHV *Rta* promoter by HIF-1α in 293 cells. The reporter plasmid pRpluc1-3087+S contains a 3-kb sequence upstream of the translational initiation site (7) and a 1-kb splicing sequence of the *Rta* gene that drives the expression of firefly luciferase. A series of truncated promoters named pRpluc1-3087, pRpluc1-2039, pRpluc1-1327, pRpluc1-941, pRpluc1-550, pRpluc550-941, pRpluc1115-1327, pRpluc1679-2039, pRplucΔ941^1679, pRplucΔ209–1115, pRplucS1-976, pRplucSΔ43^707, and pRplucS1-442 were made for the deletion of HIF-1α-binding HRE sites (the different HRE truncations are indicated by the boxed numbers). A fixed amount (5 μg) of the reporter plasmids was transfected or cotransfected with 5 μg pCEP4/HIF-1α into HEK 293 cells. Promoter activity levels are expressed as the relative light unit (RLU) induction (*n*-fold) relative to that for the reporter-alone control. The means and standard deviations from three independent transfections are shown.

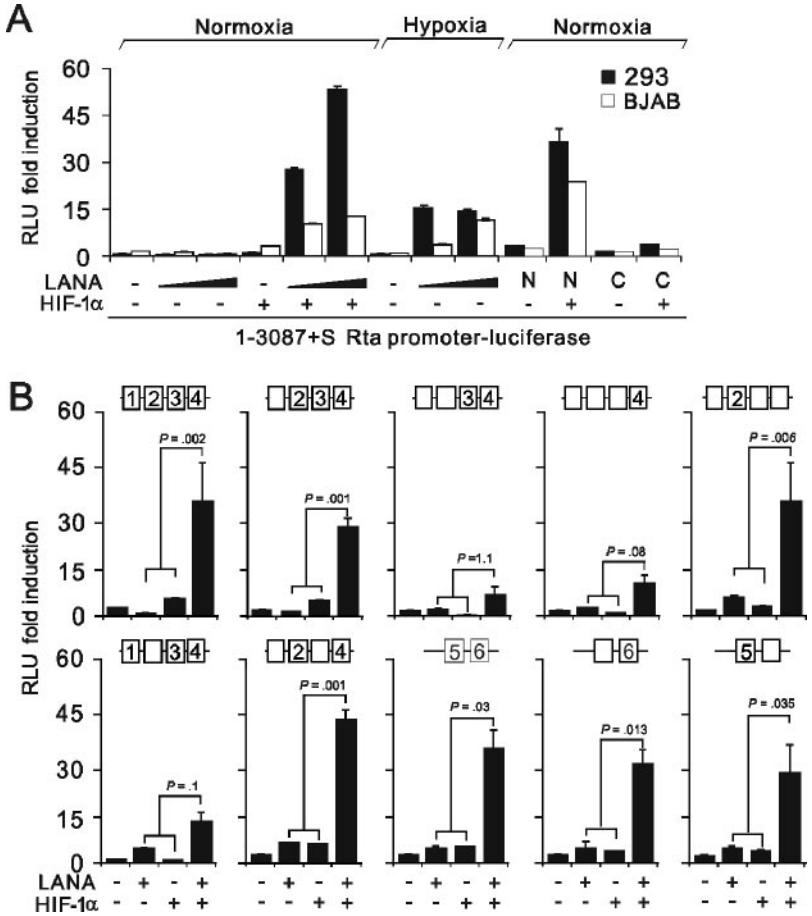

FIG. 7. The *Rta* promoter is modulated by LANA in a HIF-1α-dependent manner. (A) The cooperation of the amino domain of LANA with HIF-1α is necessary for the regulation of the *Rta* promoter. The HEK 293 or BJAB cells were transfected with 5 μg of *Rta* reporter plasmid pRpluc1-3087+S with full-length LANA or its truncations (N, amino-terminal aa 1 to 435; C, carboxyl-terminal aa 762 to 1162) in the presence or absence of HIF-1α. For normoxic and hypoxic conditions, the promoter activity levels are expressed as the relative light unit (RLU) induction (*n*-fold) relative to that for the reporter-alone control. The means and standard deviations from three independent transfections are shown. (B) HRE-2, -5, and -6 in the *Rta* promoter are critical for LANA-mediated HIF-1α regulation of RTA expression. Fixed amounts (5 μg) of *Rta* promoter reporter plasmids with different HREs truncated (indicated by the boxed numbers) were transfected into 293 cells with either pCEP4/HIF-1α (HIF-1α) or pA3M/LANA (LANA) or with both under the normoxic condition. The compared transcriptional activities of *Rta* promoter constructs with different HRE truncations in the LANA-, HIF-1α-, and LANA/HIF-1α-expressing cells are indicated by *P* values obtained by use of the Student *t* test.

nuclear extracts generated from HEK 293 cells transiently transfected with the HIF-1α expression construct. The results of the experiment show that wild-type HRE-2, HRE-5, and HRE-6 probes showed a specific shift in HIF-1α binding in nuclear extracts from HEK 293 cells overexpressing HIF-1α. The specificity of the shift was verified by the disappearance of the signal with the corresponding HRE-mutated site probe (Fig. 8A, compare lanes 2 and 4). Consistent with the reporter assay data described above, there was no detectable specific shift seen in the presence of the HRE-1, HRE-3, or HRE-4 probe (Fig. 8A).

To determine whether HIF-1α formed a complex with LANA bound to DNA, an EMSA using nuclear extract from 293 cells overexpressing HIF-1α and LANA was also performed as described above. The results showed a distinctive shift when nuclear extract was incubated with the HRE-2, HRE-5, or HRE-6 probe. The specificity of the shift was verified through its disappearance in the presence of a specific cold competitor (Fig. 8B, compare lanes 2 and 5), with no effect seen in the presence of nonspecific competitor (Fig. 8B, lanes 6). The probes were clearly supershifted in the presence of anti-HIF-1α antibodies (Fig. 8B, lanes 3). These probes were also supershifted by anti-LANA antibodies, indicating the presence of these antibodies in the complexes (Fig. 8B, lanes 4).

DISCUSSION

LANA encoded by KSHV is abundantly expressed in virus-associated malignancies (19, 30). LANA not only is critical for episomal maintenance of the viral DNA during latent infections but has also been reported to have transcriptional regulatory properties to directly modulate the promoters of cellular and viral genes, including the gene encoding hTERT (20) and its own promoters (12, 16).

Another crucial role associated with LANA's oncogenicity is

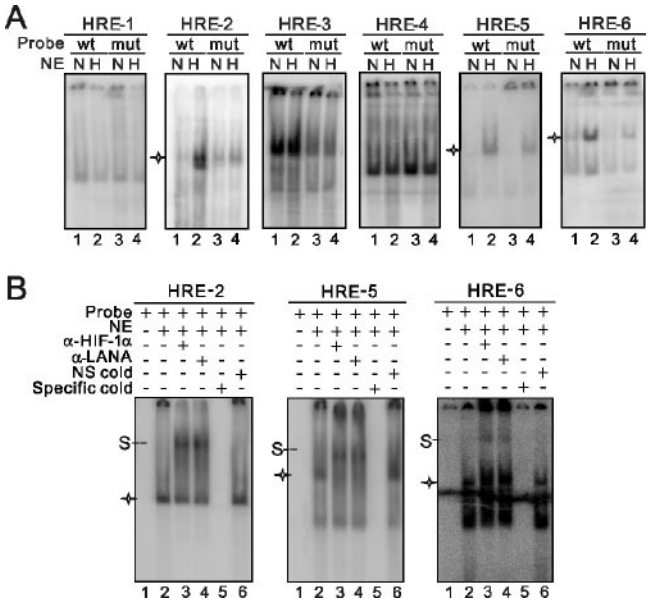

FIG. 8. LANA/HIF-1α complex binding the HREs of the KSHV *Rta* promoter. (A) HRE is involved in induction of the *Rta* promoter by HIF-1α. Nuclear extracts (NE) from HEK 293 cells transiently transfected with HIF-1α expression construct (H) or not (N) were incubated with wild-type (wt) and mutated (mut) probes (sequences are listed in Table 1) corresponding to all six putative HRE elements of the KSHV *Rta* promoter. The HIF-1α-binding complexes are indicated with diamonds. (B) HIF-1α is involved in the induction of the *Rta* promoter by LANA. For supershift analysis, the wild-type HRE-2, HRE-5, and HRE-6 probes were incubated individually with nuclear extracts from HEK 293 cells transiently cotransfected with HIF-1α and LANA expression constructs. After initial binding, the preimmune serum, anti-HIF-1α antibody, or anti-LANA antibody was added. The position of the supershift complex (S) in the presence of nuclear extract from HEK 293 overexpressing HIF-1α and LANA with specific antibodies is indicated. Excess nonlabeled specific or nonspecific (NS) probe (200 times) was used as a competitor (lanes 5).

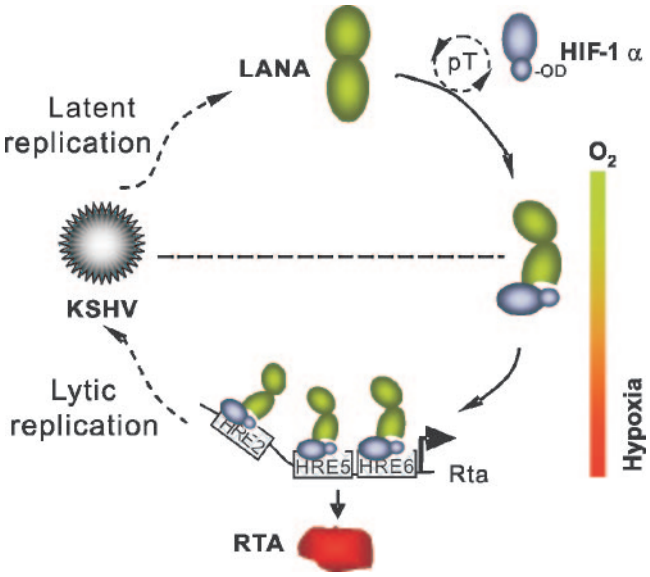

FIG. 9. Hypothetical model for KSHV-encoded latent protein LANA interaction with HIF-1α during hypoxia and KSHV reactivation. During KSHV latent replication, the essential latent protein LANA induces the transcription of the HIF-1α promoter (pT) during hypoxia. The expressed HIF-1α interacted with LANA to activate the transcription of the *Rta* promoter, which contains hypoxia response elements (HRE-2, HRE-5, and HRE-6). The highly expressed RTA protein drives virus to lytic replication. OD, oxygen-dependent domain.

its interaction with a number of transcriptional factors, such as ATF4/CREB2 (24), Sp1 (37), c-Jun (1), and STAT3 (28), to indirectly regulate downstream target gene expression. Previous studies have shown that most of these LANA-associated cellular proteins can contribute to a number of cellular processes and are involved in maintaining virus latent infection. In the present study, we have identified the amino terminal of LANA as a critical domain for KSHV response to cellular hypoxia and its interaction with HIF-1α. Specifically, we have found that the association of HIF-1α with LANA is critical for reactivating KSHV from latency to lytic replication during cellular hypoxia (Fig. 9). Functional assays demonstrated that LANA is able to enhance HIF-1α-mediated transactivation of the *Rta* gene. This indicates that LANA can also function as a coactivator in hypoxia-induced transcriptional responses to upregulate *Rta* expression (6, 13). Furthermore, these studies suggest that LANA can enhance HIF-1α transcriptional activity in human cells.

More recently, the functional interaction of complexes formed by STAT3 and LANA as well as by STAT3 and HIF-1α have been documented in two independent studies (17, 28). STAT3 was shown to function as a modulator to increase the transcriptional activity of HIF-1α in cells during hypoxia (17, 28). The prediction from these studies is that LANA may physically and functionally interact with STAT3 in PEL cells. These studies also support the probability that LANA can bind to and regulate the transcriptional activity of HIF-1α during hypoxia. Our data have verified the interaction between HIF-1α and LANA. Consistent with the multiple roles of the specific domains of LANA, the amino and carboxyl domains of LANA are associated with HIF-1α and STAT3 (17, 28) transcription factors, respectively. Thus, LANA may act as a linker to recruit these two proteins together to activate the HREs in the downstream promoters during hypoxia. However, further experimentation is needed to definitively determine this association, and we are currently pursuing these studies.

We have identified LANA as a transcriptional coactivator of HIF-1α as evidenced by the facts that (i) LANA can function as an upstream activator of *Rta* in a HIF-1α-dependent fashion, (ii) LANA bound to HRE elements in vitro, (iii) LANA colocalized with HIF-1α in cells, and (iv) LANA interacted with HIF-1α in coimmunoprecipitation assays. In addition, LANA induces HIF-1α protein expression in a dose-dependent manner, similar to the Epstein-Barr virus latent oncoprotein LMP1 (38).

Previous studies in our lab have shown that a likely mechanism for KSHV maintaining latent infection is the association of LANA with RBP-Jκ protein to repress RTA expression (22). Here, we address another potential mechanism for virus reactivation from latency to lytic replication, in which LANA cooperates with HIF-1α to promote RTA expression during hypoxia. These two distinct mechanisms manipulated by the KSHV major latent protein LANA for controlling latency or

lytic replication indicate that KSHV can usurp different cellular mechanisms to regulate its progression in host cells under different environmental conditions, such as regular and low oxygen conditions. Earlier studies have revealed that HIF-1α as well as HIF-2α can contribute to the activation of the *Rta* promoter (13). This indicates that multiple hypoxia-inducible factors are probably involved in regulating *Rta* expression during cellular hypoxia. The possibility of LANA being associated with HIF-2α protein does exist and requires further studies to determine the potential role of this and other interactions.

To elucidate the molecular mechanisms for KSHV involved in hypoxic induction, we analyzed the upstream promoter region including the intron region of its essential lytic gene *Rta*, which is different from that found by Haque and colleagues, who focused only on the upstream regulatory *Rta* promoter region (13). Meanwhile, three potential HIF-1α-binding consensus sequences were used to scan the regulatory sequences in the upstream promoter as well as within the intron (9, 21, 27, 33). We have found six potential HRE elements within these regions and identified three functional hypoxia-responsive elements: HRE-2, located at positions −1130 to −1123 upstream of the transcriptional start site; HRE-5, located at positions +234 to +241 within the intron sequence downstream of the transcriptional start site; and HRE-6, also located within the intron at positions +812 to +820 (7). The results of the luciferase reporter assays showed that these elements were necessary and sufficient for HIF-1α-mediated activation of the *Rta* promoter. Subsequent analysis by EMSA revealed that a hypoxia-inducible protein complex containing HIF-1α and LANA interacted with the HRE-2, -5, and -6 elements. However, it is noteworthy that the transcriptional activity of the reporter construct pRplucΔ209-1115, which contains the HRE-2, -3, and -4 motifs, was dramatically increased compared to that of the reporter construct pRpluc1-1327 and compared to that of the reporter construct characterized by a deletion of the HRE-3 motif at positions −290 to −941. The transcriptional factor binding site analysis of this region showed that there are three constitutive GATA-1-binding sites. This suggests that the transcriptional repressor GATA-1 is likely to be involved in regulation of the *Rta* promoter in cooperation with LANA. Furthermore, the transcription activity of the construct pRplucΔ209-1115, which excludes the GATA-1-binding sites coexpressed with LANA, was significantly activated strongly, supporting the argument for cooperation with LANA.

In summary, our results indicate that LANA enhances the expression of RTA through a HIF-1α-dependent induction of hypoxia (Fig. 9). Activation of this cascade is important for KSHV response to hypoxia and the start of lytic replication in the hypoxic microenvironment. However, it is likely that RTA does not solely rely on hypoxia for reactivation from latency but may also utilize other cellular cues. Our current results provide some clues to understanding the underlying mechanism by which hypoxia can induce KSHV lytic replication from latency.

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