Kruppel-Associated Box Domain-Associated Protein-1 as a Latency Regulator for Kaposi's Sarcoma-Associated Herpesvirus and Its Modulation by the Viral Protein Kinase

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) has been linked to the development of Kaposi's sarcoma, a major AIDS-associated malignancy, and to hematologic malignancies, including primary effusion lymphoma and multicentric Castleman's disease. Like other herpesviruses, KSHV is capable of both latent and lytic replication. Understanding the molecular details associated with this transition from latency to lytic replication is key to controlling virus spread and can affect the development of intervention strategies. Here, we report that Kruppel-associated box domain-associated protein-1 (KAP-1)/transcriptional intermediary factor 1β, a cellular transcriptional repressor that controls chromosomal remodeling, participates in the process of switching viral latency to lytic replication. Knockdown of KAP-1 by small interfering RNA leads to KSHV reactivation mediated by K-Rta, a key transcriptional regulator. In cells harboring latent KSHV, KAP-1 was associated with the majority of viral lytic-gene promoters. K-Rta overexpression induced the viral lytic cycle with concomitant reduction of KAP-1 binding to viral promoters. Association of KAP-1 with heterochromatin was modulated by both sumoylation and phoshorylation. During lytic replication of KSHV, KAP-1 was phosphorylated at Ser^{824}. Several lines of evidence directly linked the viral protein kinase to this post-translational modification. Additional studies showed that this phosphorylation of KAP-1 produced a decrease in its sumoylation, consequently decreasing the ability of KAP-1 to condense chromatin on viral promoters. In summary, the cellular transcriptional repressor KAP-1 plays a role in regulating KSHV latency, and viral protein kinase modulates the chromatin remodeling function of this repressor. [Cancer Res 2009;69(14):5681–9]

Introduction

The Kruppel-associated box domain-associated protein-1 (KAP-1)/transcriptional intermediary factor 1β was initially identified as a universal transcriptional corepressor for Kruppel-associated box domain-containing zinc finger protein, the largest family of transcriptional silencers in the human genome (1). KAP-1 recruits and coordinates the assembly of several chromatin-remodeling proteins, such as histone deacetylase multiprotein complexes (e.g., nucleosome remodeling and deacetylase and nuclear receptor corepressor), histone methyltransferases [e.g., SET domain, bifurcated 1 (SETDB1)], and heterochromatin protein 1 (HP1) through its plant homeodomain, bromodomain, and PXVXL motif (2–5). These proteins, together with trimethylated histone 3 Lys^{9} (H3K9m3) and histone 3 Lys^{27} (H3K27m3), are hallmarks of heterochromatin. As a corepressor, KAP-1 interacts with murine double minute 2, melanoma antigen, and signal transducer and activator of transcription 3, thereby modulating transcriptional activity of p53 and signal transducer and activator of transcription 3 (6–8). Increasing evidence suggests that post-translational modifications, such as phosphorylation and sumoylation, are important for regulating the repression function of KAP-1 (9). Phosphorylation of KAP-1 at Ser^{824} by phosphatidylinositol 3-kinase-like protein kinases, such as ataxia telangiectasia mutated (ATM), is critical to chromatin relaxation in response to DNA damage (10, 11). Sumoylation of KAP-1 at Lys^{554}, Lys^{779}, and Lys^{804} generates binding platforms for SETDB1 and histone deacetylase 1, thus enhancing the corepression function of KAP-1 (12–14). Importantly, phosphorylation of Ser^{824} is antagonistic to sumoylation at these three sites. Thus, these post-translational modifications affect the ability of KAP-1 to condense or relax chromatin (9).

A common property of herpesviruses is their capacity to establish latency, whereby the majority of viral genes are silenced and the genome persists in cells as an episome, which is maintained in a condensed chromatin state. On induction by certain viral gene products or chemicals, the viral episosme gradually relaxes its compact chromatin structure, leading to expression of all viral genes and lytic replication. Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is an oncogenic γ-herpesvirus involved in the pathogenesis of Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease (15, 16). Like other herpesviruses, KSHV undergoes both lytic and latent infections, with strong tendency toward the latter (17). Latent to lytic transition can be triggered by a single viral gene product, K-Rta (18), or by chemicals that activate cell signaling pathways and influence chromatin structure, such as phorbol esters and butyrate (19). These findings indicate that this transition involves global remodeling of viral chromatin from the heterochromatin to the euchromatin state (20). Although K-Rta functions to switch from latency to lytic replication, additional early viral gene products are also important for optimal transcription of the viral episome during the lytic phase of replication. The exact sequence of events associated with chromosomal remodeling of KSHV is not well understood. Given the prominent role of KAP-1 in regulation of heterochromatin formation, we explored its role in

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the life cycle of KSHV. Using two different cell culture systems as models for KSHV reactivation, we showed that modulation of KAP-1 levels exhibited a profound effect on maintaining viral latency. Our studies also showed that viral protein kinase (vPK/ORF36) phosphorylates KAP-1 and thereby modulates its chromatin remodeling activity.

Materials and Methods

Cell culture and plasmid DNA. 293, 293T, TREx-F3H3-K-Rta, and TREx-F3H3-vPK BCBL-1 cells were cultured as described previously (21). KAP-1 knockdown cell lines were generated by transduction of TREx-F3H3-K-Rta BCBL-1 cells with lentiviral particles expressing short hairpin RNA (shRNA) targeting KAP-1 (*CCGG*GAGGACTACAACCTTATTGTT*CTCGAG*AACAAAATAAGGTTGTAGTCCTC*TTTTT*) and then selected with puromycin (1 μg/mL; Invitrogen). To overexpress wild-type KAP-1 and its mutant in KAP-1 knockdown cells, shRNA-resistant KAP-1 construct was developed by mutating the shKAP-1 target site to GAGGACTACAATTTGATTGTT. Expression vectors containing shRNA-resistant KAP-1* and KAP-1*-S824D were transiently transfected into cells using FugeneHD and selected with G418 (150 μg/mL; Cellgro). The Vero-rKSHV.219 cell line was kindly provided by Dr. Jeffrey Vieira (University of Washington; ref. 22). Expression vectors pCMV-Tag2A-Flag-KAP-1 (9), pcDNA3-T7-wild-type vPK (vPK-wt), and pcDNA3-T7-vPK-K108Q were described previously (23). pGL3-Basic (Promega) and pGEX-2T (Amersham Pharmacia Biotech) were used to construct reporter and GST fusion proteins. The pLK0.1 and pLK0.1-shKAP-1 vectors (Open Biosystems) were used to generate lentivirus expressing KAP-1 shRNA.

Immunoprecipitation and Western blot analysis. Nuclear extracts of TREx-F3H3-vPK BCBL-1 cells were prepared as described previously (2). Transfected 293 cells were collected in modified radioimmunoprecipitation assay buffer (24). Cell lysates were incubated with anti-Flag agarose (Sigma) overnight at 4°C. Agarose were washed and proteins were analyzed by immunoblotting. The following antibodies were used: anti-Flag and

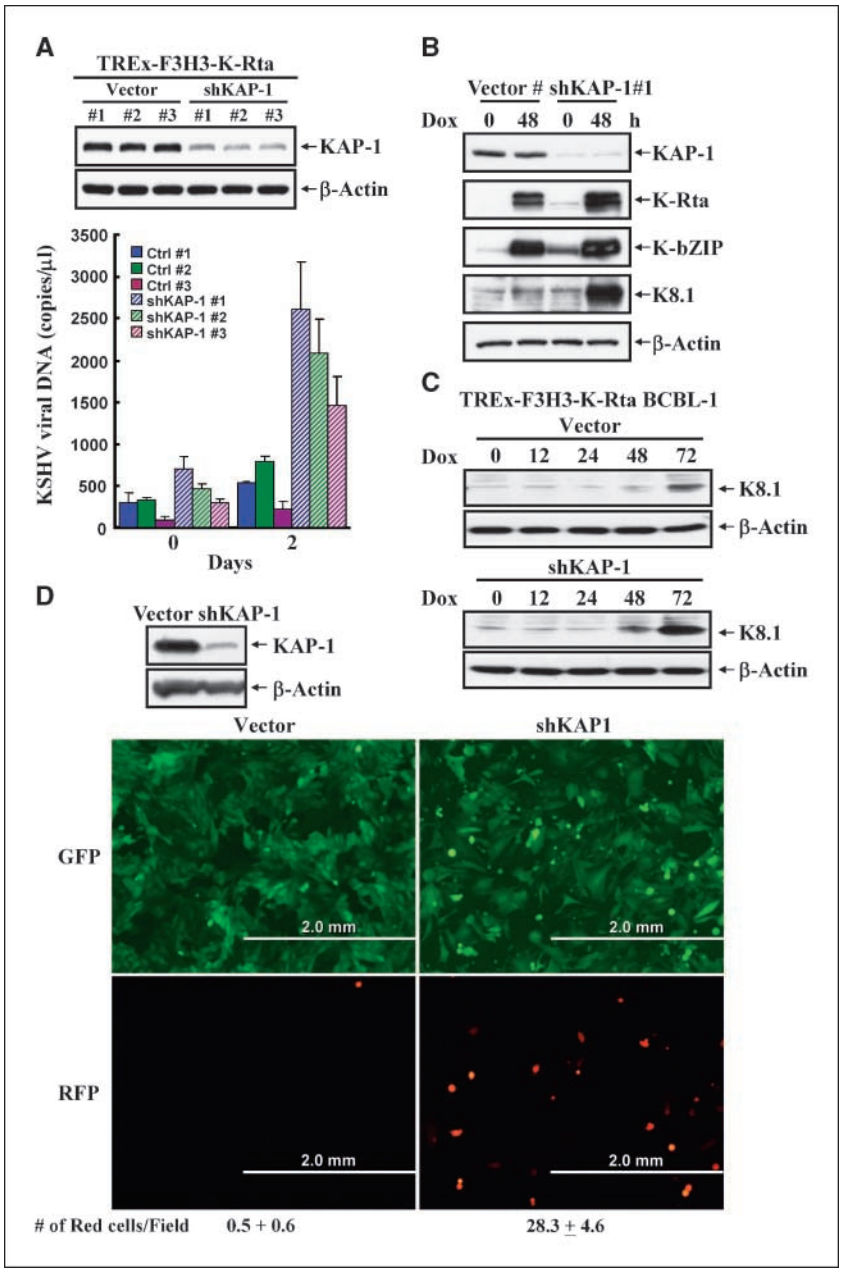


Figure 1. KAP-1 knockdown increases KSHV replication and lytic gene expression. *A,* total cell lysates from vector-control and KAP-1 knockdown TREx-F3H3-K-Rta BCBL-1 cells were analyzed by immunoblotting. Supernatants were collected at days 0 and 2 after doxycycline (*Dox*; 0.1 μg/mL) induction and levels of virion-associated DNA were determined by real-time quantitative PCR. Mean ± SD. *B,* total cell lysates from the cells described above were immunoblotted. *C,* total cell lysates were collected from vector-control and KAP-1 knockdown BCBL-1 cells at 0, 12, 24, 48, and 72 h after doxycycline (0.1 μg/mL) induction and immunoblotted with anti-K8.1 antibody. *D,* total cell lysates from Vero-rKSHV.219 cells, transduced by control and KAP-1 shRNA lentiviruses, were analyzed by immunoblotting. *Left,* vector-control cells; *right,* shKAP-1 knockdown cells; *top,* GFP fluorescence (latent-infected cells); *bottom,* red fluorescent protein (*RFP*) fluorescence (lytic-infected cells).

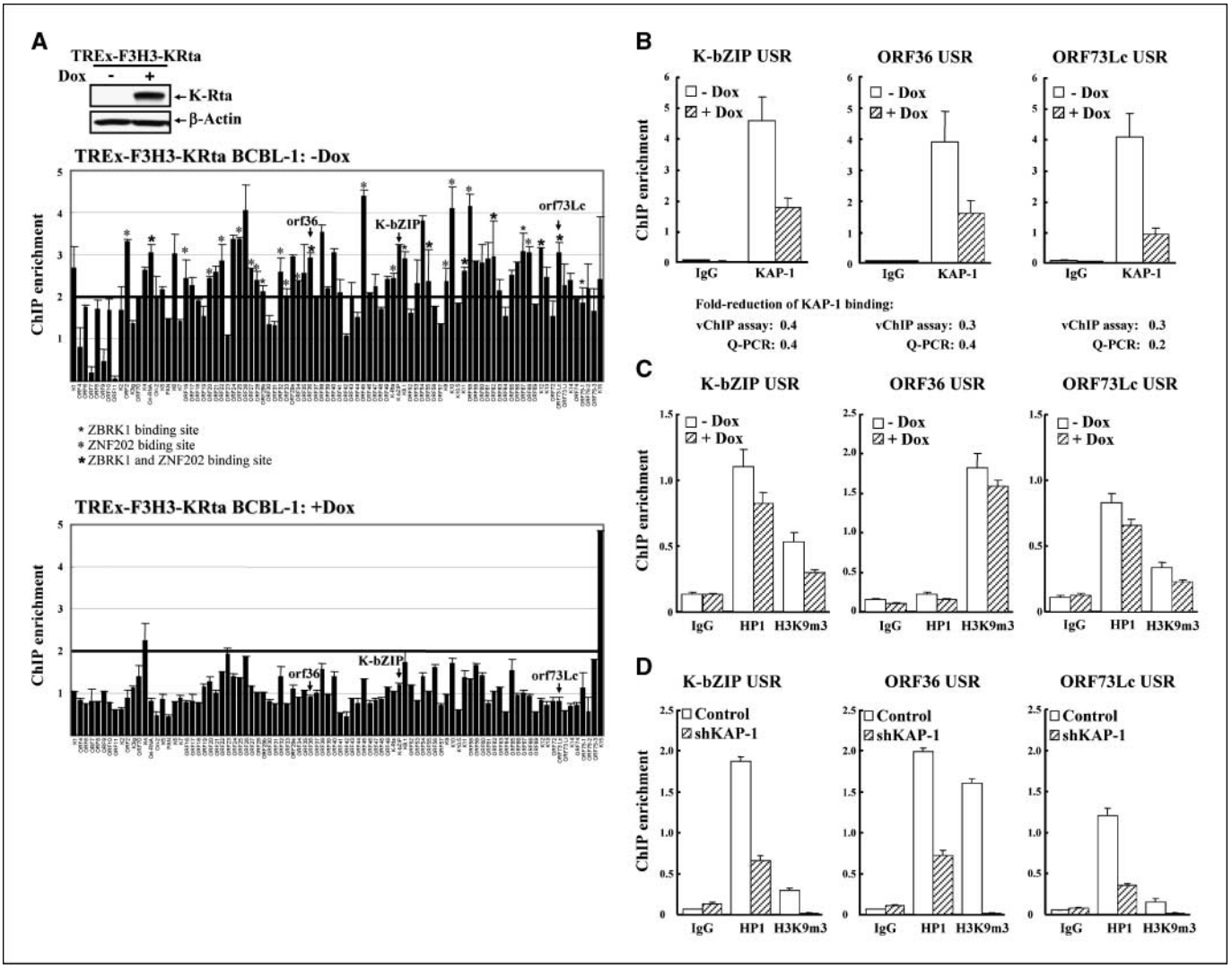


Figure 2. Chromatin immunoprecipitation assay to determine KAP-1, HP1, and H3K9m3 binding to KSHV promoters during viral reactivation. *A,* immunoblotting of K-Rta expression in TREx-F3H3-K-Rta BCBL-1 cells treated with doxycycline (0.1 μg/mL) for 24 h. β-Actin was used as control. Chromatin immunoprecipitation (*ChIP*) assay was done on noninduced and K-Rta–induced BCBL-1 cells using anti-KAP-1 antibody or IgG. KAP-1 binding pattern along KSHV promoters before (*middle*) and after (*bottom*) viral reactivation. Mean ± SD of replicate spots. *B,* ChIP-on-vChip data were verified by real-time quantitative PCR using primers specific for USR of KSHV *K-bZIP, vPK/ORF36,* and *LANA/ORF73Lc*. Input DNA was normalized to 1. Mean ± SD. *C,* chromatin immunoprecipitation assay was done on noninduced and K-Rta–induced BCBL-1 cells using IgG, anti-HP1, or anti-H3K9m3 antibody. Chromatin immunoprecipitation DNA was analyzed as described in *B. D,* analysis of chromatin immunoprecipitation DNA of HP1 and H3K9m3 bound to KSHV promoters in control and KAP-1 knockdown BCBL-1 cells was done as described in *B*.

anti-β-actin (Sigma), anti-KAP-1 (Cell Signaling Technologies), anti-phospho-KAP-1(Ser^{824}) (Bethyl Laboratories), and anti-vPK antibody (24).

Mass spectrometry analysis of vPK-interacting proteins. TREx-F3H3-vPK BCBL-1 cells were treated with doxycycline (0.1 μg/mL) to induce vPK expression. Nuclear extracts were prepared from vPK-inducible BCBL-1 cells at 48 h after doxycycline treatment. vPK-interacting protein complexes were coimmunoprecipitated using anti-Flag agarose and eluted by flag peptide. Eluted complexes were separated by 6% to 14% SDS-PAGE, detected by Coomassie blue staining, and identified using liquid chromatography-tandem mass spectrometry (Protein and Nucleic Acid Facility, Stanford University).

Reporter assay. 293 cells were cultured in 24-well plates. Plasmids were transfected using TransFectin (Bio-Rad). Forty-eight hours after transfection, cell lysate was prepared and luciferase activity was determined by the Luciferase Assay System (Promega).

***In vitro* protein kinase assay.** vPK kinase activity was measured as described previously (24); purified vPK-wt or kinase-dead vPK-K108Q (0.1 μg) were incubated with GST-KAP-1 or GST-KAP-1-S824A substrates.

ChIP-on-vChip assay. Chromatin immunoprecipitation assay was done according to a previous protocol.[4] Antibodies used were anti-KAP-1 (Abcam), anti-HP1α (Upstate), anti-H3K9m3 (Abcam), and rabbit IgG (Alpha Diagnostic International). Chromatin immunoprecipitation DNA and 10% input were amplified using a whole-genome amplification kit (Sigma). Chromatin immunoprecipitation sample was labeled with Cy3, and input sample was labeled with Cy5 using the 3DNA array 900DNA kit (Genisphere). After cohybridization of labeled DNA samples to the viral chip, the slides were scanned with the Agilent DNA microarray scanner at a resolution of 10 μm. Images were captured and quantified using Scanalyze software.[5] The chromatin immunoprecipitation signal of the experimental sample was normalized and compared with control input.

[4] http://genomics.ucdavis.edu/farnham
[5] http://rana.lbl.gov/EisenSoftware.htm

Assays of KSHV growth and gene expression in KAP-1 knockdown BCBL-1 cells. To assess viral growth, supernatant from 7.5×10^5 of control and doxycycline-induced (0.2 μg/mL) KAP-1 knockdown, small interfering RNA-resistant KAP-1 and KAP-1-S824A overexpressed TREx-F3H3-K-Rta and TREx-F3H3-vPK BCBL-1 cells were collected at 0 and 48 h. DNA from virions was prepared (25) and quantified by real-time PCR (TaqMan) as described previously (17). For measuring viral protein expression, total cell lysates were prepared at 0 and 48 h after K-Rta induction and immunoblotted.

Results

KAP-1 knockdown enhances KSHV replication. To explore the role of KAP-1 in KSHV reactivation, we stably expressed KAP-1 shRNA in a BCBL-1 cell line carrying the Tet-inducible K-Rta viral transactivator (TREx-F3H3-K-Rta BCBL-1; ref. 21). Mixed populations of puromycin-resistant cells were isolated, and knockdown of KAP-1 was assessed by immunoblotting (Fig. 1*A, top*). To determine the effect of KAP-1 on production of virus, supernatants were collected after doxycycline induction of K-Rta and analyzed for levels of virion-associated DNA by quantitative PCR amplification. KAP-1 knockdown significantly enhanced viral reactivation and increased virus production by ~5-fold over vector-control cells at 48 h after induction (Fig. 1*A, bottom*). In agreement with increased virus release, levels of immediate-early/early genes, K-Rta and K-bZIP, and late gene, K8.1, were elevated in KAP-1 knockdown cells (Fig. 1*B*). The effect of KAP-1 knockdown was also examined in the Vero-rKSHV.219 cell line, which harbors recombinant KSHV genome expressing green fluorescent protein (GFP) from the cellular *EF-1α* promoter and red fluorescent protein from the viral lytic *PAN* promoter. All cells expressed GFP with very few producing red fluorescent protein, indicating tight control of latency. However, in KAP-1 knockdown cells, red fluorescent protein-positive cells increased 25- to 50-fold (Fig. 1*D*). These data suggest that KAP-1 acts to suppress lytic replication presumably by compacting and silencing viral chromatin.

KAP-1 is associated with latent KSHV genomes and dissociates after reactivation. To determine whether KAP-1 binds to KSHV chromatin, we performed a ChIP-on-vChip assay by hybridizing DNA from KAP-1 associated viral chromatin with a viral promoter array chip (vChip) designed by our laboratory (26). This array contains upstream sequence regions (USR) of 83 KSHV genes; each USR, ~500 bp, contains sites for assembly of transcription factors (26). Cross-linked viral chromatin was prepared by sonication of uninduced and doxycycline-induced TREx-F3H3-K-Rta BCBL-1 cells. Expression of K-Rta was verified by immunoblot (Fig. 2*A, top*). Chromatin was immunoprecipitated from cell lysates with anti-KAP-1 antibody. To verify this approach, we first showed that KAP-1 binds the *ZNF433* promoter, a known KAP-1 target (ref. 27; data not shown). DNA from KAP-1 immunoprecipitates and input were hybridized on the vChip. The enrichment value of KAP-1 binding to promoters was obtained by dividing normalized levels of immunoprecipitated DNA intensity by the input DNA for each array spot. Taking 2-fold difference as significant, KAP-1 was associated with more than two-thirds of viral USRs in uninduced cells (Fig. 2*A, middle*), whereas only two USRs scored significantly after K-Rta induction for 24 h (Fig. 2*A, bottom*). This finding suggests that KAP-1 associates with latent viral chromatin and dissociates during reactivation. The ChIP-on-vChip data were confirmed by gene-specific real-time quantitative PCR using three USRs representing early genes (*K-bZIP*/*K8* and *vPK*/*ORF36*) and latent genes (*LANA*/*ORF73*). Consistent with our vChip data, KAP-1 bound to transcription complexes assembled at all three promoters before K-Rta induction and dissociated after reactivation (Fig. 2*B*).

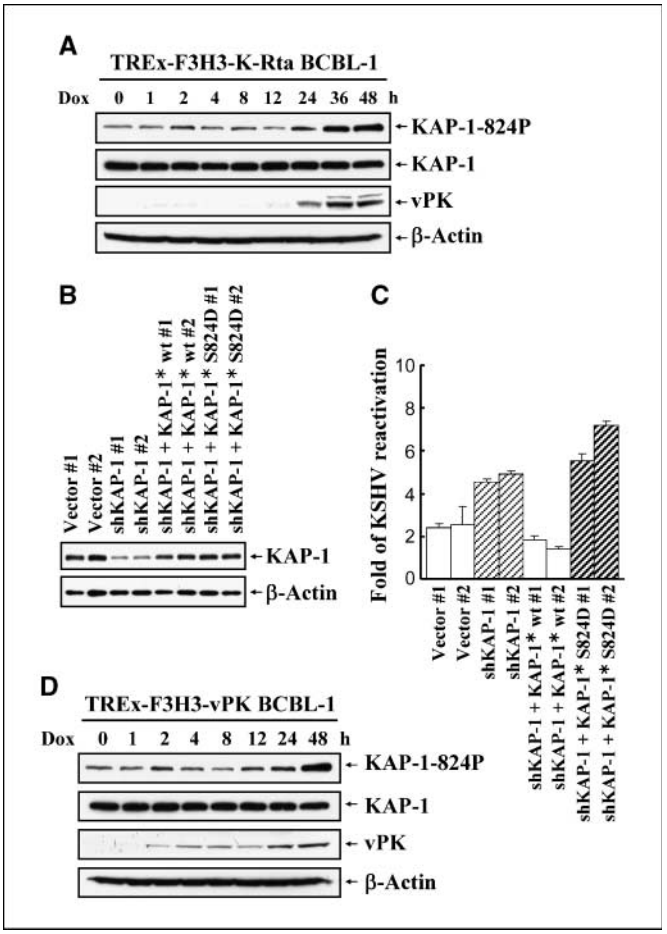


Figure 3. Phosphorylation of KAP-1 Ser^{824} site plays an essential role in KSHV reactivation. *A,* phosphorylation of KAP-1 Ser^{824} site during KSHV reactivation. TREx-F3H3-K-Rta BCBL-1 cells were treated with doxycycline (0.1 μg/mL) and harvested at the indicated time points shown for phospho-Ser^{824} KAP-1, KAP-1, vPK, and β-actin immunoblots. *B,* immunoblotting of KAP-1 expression in vector-control, KAP-1 knockdown, and shRNA-resistant KAP-1 and KAP-1-S824D mutant transfected TREx-F3H3-K-Rta BCBL-1 cells. *C,* virion-associated DNA from supernatants of cells described in *B* were collected at days 0 and 2 after doxycycline (0.1 μg/mL) induction and determined as described in Fig. 1*A*. KSHV reactivation at day 2 was normalized against day 0. *D,* phosphorylation of KAP-1(Ser^{824}) during vPK overexpression. TREx-F3H3-vPK BCBL-1 cells were treated and analyzed as in *A*.

Effect of KAP-1 dissociation on KSHV heterochromatin formation. Heterochromatin is characterized by high levels of H3K9m3 and corecruitment of HP1. KAP-1 interacts with HP1 and SETDB1, which is a H3K9m3 methylase. Accordingly, KAP-1 can facilitate formation of heterochromatin (28). We determined whether HP1 association or H3K9m3 changed during viral reactivation as predicted by this model. TREx-F3H3-K-Rta BCBL-1 cells were used for analysis of transcription complexes assembled on the USRs of *K-bZIP, vPK,* and *LANA*. A general trend of decreasing HP1 and H3K9m3 association was observed 24 h after K-Rta induction. Although the decrease in the *vPK* USR was marginal, the reduction of H3K9m3 binding to these USRs was accompanied by an increase in H3K9Ac as we recently reported (ref. 26; Fig. 2*C*). To test whether KAP-1 down-modulation is, in part, responsible for decreasing HP1 and H3K9m3 association, we examined HP1 and H3K9m3 binding levels on these three USRs using KAP-1 sh

RNA-transduced TREx-F3H3-K-Rta BCBL-1 cells. KAP-1 knockdown significantly lowered HP1 and K3K9m3 levels in all three regions (Fig. 2*D*).

Role of KAP-1 Ser^{824} phosphorylation in viral reactivation. To explore the mechanism by which KAP-1 modulates viral reactivation, levels of KAP-1 and KAP-1(Ser^{824}) phosphorylation were monitored. Ser^{824} phosphorylation by ATM is of particular interest because this post-translational modification is responsible for relaxing chromatin that surrounds sites of DNA damage (10, 11). Levels of KAP-1(Ser^{824}) phosphorylation increased after 24 h of induction, whereas KAP-1 levels remained unchanged (Fig. 3*A*). To test the hypothesis that KAP-1(Ser^{824}) phosphorylation plays a role in maintaining open viral chromatin, we developed a shRNA-resistant KAP-1*-S824D, which mimics KAP-1 phosphorylation. This mutant, as well as shRNA-resistant KAP-1*, was introduced into the KAP-1 knockdown TREx-F3H3-K-Rta BCBL-1 cell line. Expression of each of these constructs was confirmed in transfected cells (Fig. 3*B*). As before, depleting KAP-1 by shRNA increased lytic replication at 48 h after K-Rta induction (Fig. 3*C*). Introduction of shRNA-resistant wild-type KAP-1* restored lytic replication to original levels. By contrast, shRNA-resistant KAP-1-S824D lacked the ability to repress lytic replication and instead further enhanced release of virus. These data taken together suggest that phosphorylation of KAP-1 at Ser^{824} affects its ability to repress chromatin.

vPK interacts with and phosphorylates KAP-1 at Ser^{824}. The correlation of KAP-1 phosphorylation with vPK expression prompted us to investigate whether vPK may modulate KAP-1 activity. A Flag-HA-tagged, vPK-inducible BCBL-1 cell line was generated (TREx-F3H3-vPK BCBL-1). During vPK induction, phosphorylation of KAP-1(Ser^{824}) increased, whereas steady-state levels of KAP-1 remained unchanged (Fig. 3*D*). This finding suggests that vPK induces phosphorylation of KAP-1, either directly or indirectly. Taking advantage of the doubly tagged vPK in conjunction with tandem mass spectroscopy, we identified proteins associated with affinity-purified vPK. Interestingly, KAP-1 was among the major proteins associated with vPK (Fig. 4*A*). Other cellular proteins identified by this methods included heat shock protein chaperones, which are known to interact with protein kinases. To confirm this interaction, 293T cells were cotransfected with T7-vPK and Flag-KAP-1 or Flag-vPK alone. Transfected proteins were immunoprecipitated using anti-Flag agarose. Immunoblotting of KAP-1 and vPK complexes confirmed the interaction of these two proteins (Fig. 4*B*). This interaction was also shown in naturally infected BCBL-1 cells after K-Rta induction (Fig. 4*C*). Together, these data showed that KAP-1 and vPK form a protein complex.

To determine whether vPK directly phosphorylated KAP-1, we performed an *in vitro* kinase reaction using recombinant purified vPK-wt and kinase-dead vPK-K108Q, with GST-KAP-1 substrate. vPK-wt readily phosphorylated KAP-1, whereas kinase-dead mutant did not (Fig. 5*A, left*). The data described previously imply that vPK phosphorylates KAP-1(Ser^{824}). *In vitro* kinase assay was done using either wild-type KAP-1 or KAP-1-S824A. The results show that KAP-1 phosphorylation was reduced in the S824A mutant compared with wild-type (Fig. 5*A, right*), indicating that Ser^{824} is a major phosphorylation site for vPK. Residual phosphorylation of the KAP-1 mutant indicates additional sites of vPK phosphorylation. To further show that this phosphorylation occurs *in vivo*, Flag-KAP-1 or Flag-KAP-1-S824A were cotransfected with vPK-wt or K108Q into 293 cells. vPK phosphorylation of KAP-1 was monitored by immunoblot using anti-phospho-KAP-1(Ser^{824}) antibody. Consistent with the *in vitro* results, vPK phosphorylated wild-type KAP-1 at Ser^{824} but not its S824A mutant *in vivo* (Fig. 5*B*).

Sumoylation of KAP-1 is affected by vPK. KAP-1 trans-repression and recruitment of SETDB1 histone methylase depends

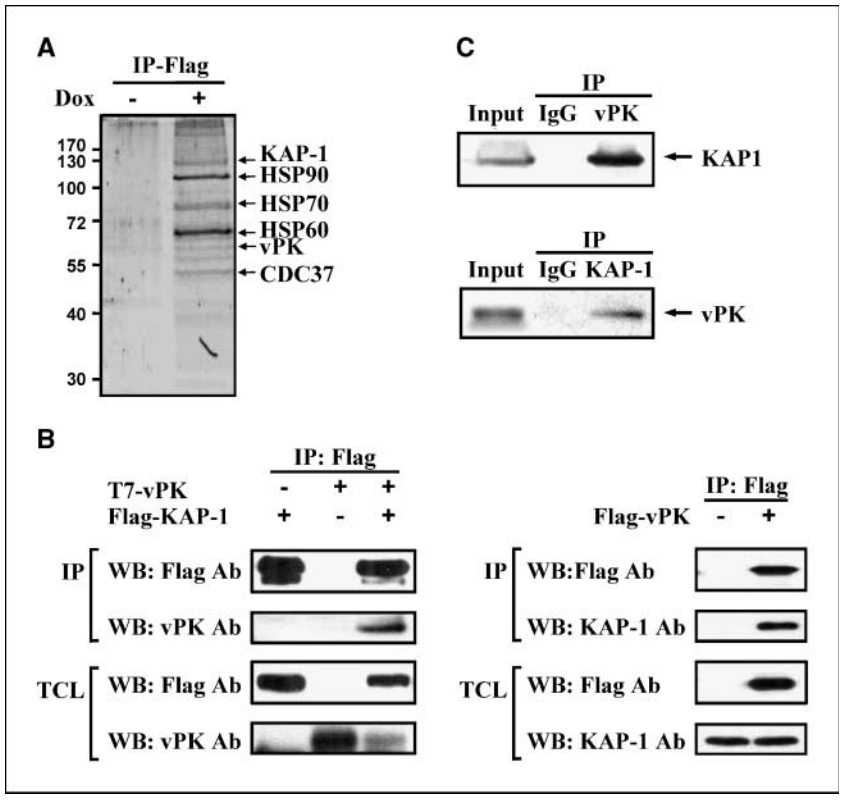
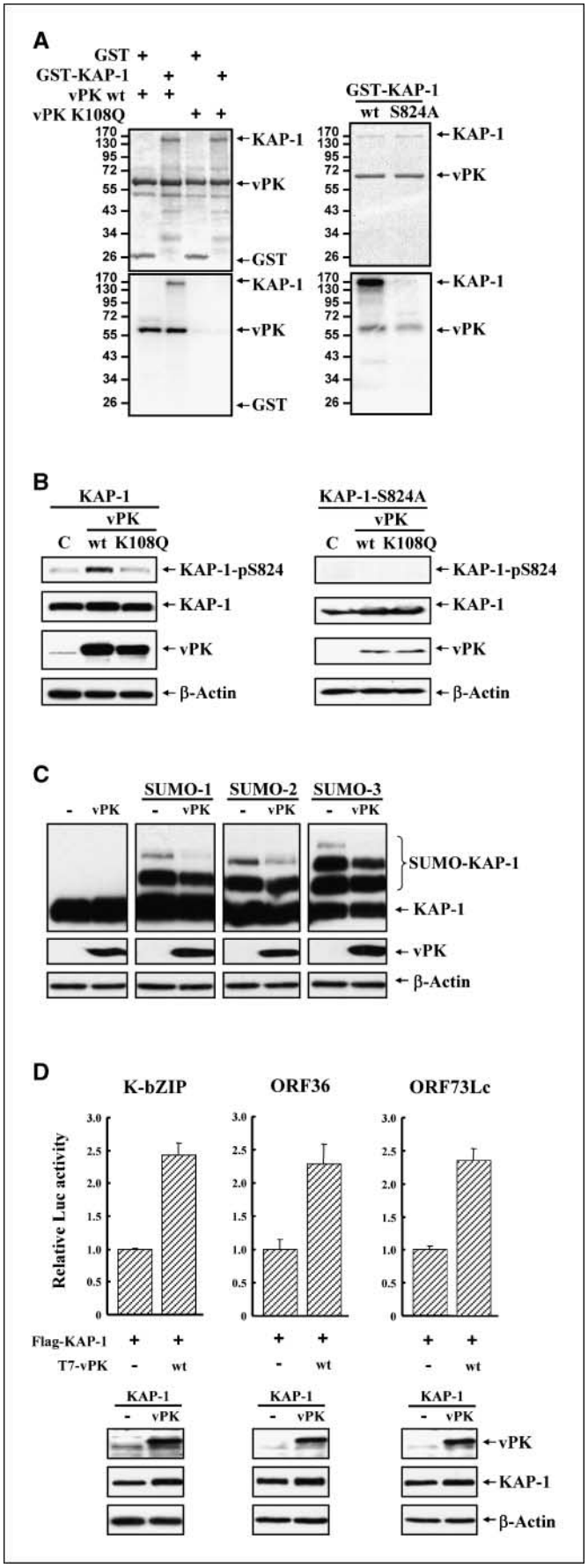


Figure 4. KAP-1 forms a complex with vPK. *A,* Coomassie blue staining of vPK-interacting proteins. *B,* 293T cells were transiently transfected with expression vectors encoding Flag-tagged KAP-1, T7-tagged-vPK, or both (*left*) or Flag-tagged vPK (*right*). Flag-KAP-1 or Flag-vPK was immunoprecipitated with anti-Flag agarose followed by immunoblotting with the indicated antibodies. Total cell lysates (*TCL*) were probed for protein expression. *C,* total cell lysates from noninduced and K-Rta–induced BCBL-1 cells were subjected to immunoprecipitation with anti-KAP-1, anti-vPK antibody, or IgG followed by immunoblotting with the indicated antibodies.



on its sumoylation status, whereas Ser^{824} phosphorylation reduces the degree of KAP-1 sumoylation (9, 13). Accordingly, we asked whether vPK also modulated KAP-1 sumoylation as well as its transcriptional functions. Flag-KAP-1 and T7-vPK were cotransfected into 293 cells together with plasmids expressing three SUMO species. vPK reduced the extent of KAP-1 sumoylation in all these settings (Fig. 5*C*). In parallel, we examined whether vPK affects KAP-1-mediated transcriptional repression by reporter assay using three KSHV promoters. The repressive effect of KAP-1 on *K-bZIP*, *ORF36*, and *ORF73Lc* promoters was relieved by vPK-wt (Fig. 5*D*).

vPK enhances KSHV viral replication. Because KAP-1 regulates KSHV latency and vPK modulates KAP-1 activity via post-translational modification, the affect of vPK on regulation of KSHV latency was investigated. Using the vPK-inducible BCBL-1 cell lines, we tested whether levels of spontaneous or induced reactivation increased on vPK induction. When vPK was induced by doxycycline, virion-associated DNA in the supernatant at 48 h after induction was ~3-fold higher compared with the controls (Fig. 6*A, left*). Western blot analysis confirmed higher expression levels of K-Rta, K-bZIP, and K8.1 in the vPK-induced cells (Fig. 6*A, right*), further suggesting that vPK enhances viral reactivation. Next, we determined whether vPK could also augment chemically induced viral reactivation with either TPA or TSA. TPA functions through activation of the protein kinase C pathway, whereas TSA inhibits histone deacetylase. Interestingly, vPK overexpression synergizes with TPA, but not TSA, for induction of virus production (Fig. 6*B* and *C*). The above observation suggests that vPK-mediated reactivation uses the histone acetylation pathway.

Conversely, we determined whether knockdown of vPK would decrease viral production in the reactivation model. Using the Vero-rKSHV.219 cell line, which allows high efficiency of shRNA delivery, we tested whether vPK knockdown reduces virus production. In this experiment, K-Rta and shRNA-vPK were cotransfected, and levels of extracellular virion DNA were measured. Introduction of shRNA-vPK reduced viral titer to ~40% to 60% of the K-Rta-induced value in comparison with shRNA against GFP; these findings further support that vPK plays a role in facilitating viral reactivation (Fig. 6*D*).

Discussion

A common feature of oncogenic viruses is their ability to enter latency. Understanding viral and cellular factors involved in establishment and maintenance of latency can provide insight into intervention strategies targeting malignancies associated with these viruses. Increasing evidence suggests that chromatinization and histone modification play important roles in regulating viral latency (20, 29). During latency, the herpesvirus genome is maintained as an

Figure 5. vPK phosphorylates KAP-1, reduces KAP-1 sumoylation, and alleviates KAP-1 transcriptional repression function. *A, left,* purified GST or GST-KAP-1 was incubated with vPK-wt or vPK-vPK-K108Q in the *in vitro* kinase reaction. Phosphorylated substrates were resolved on SDS-PAGE and detected by autoradiography. Equal amounts of input proteins were confirmed by Coomassie blue staining. *Right, in vitro* kinase assay was done as described using GST-KAP-1 and GST-KAP-1-S824A. *B,* control or vector expressing T7-vPK or T7-vPK-K108Q was cotransfected with Flag-KAP-1 or Flag-KAP-1-S824A, and total cell lysate was immunoblotted with the indicated antibodies. *C,* 293 cells were cotransfected with T7-vPK-wt, Flag-KAP-1, and T7-SUMO-1, T7-SUMO-2, or T7-SUMO-3. Forty-eight hours post-transfection, total cell lysates were separated by SDS-PAGE and immunoblotted using specific antibodies. *D,* 293 cells were cotransfected with KSHV *K-bZIP*, *vPK*/*ORF36*, or *LANA*/*ORF73Lc* promoter luciferase reporter and T7-vPK-wt together with Flag-KAP-1. After 48 h, luciferase activities were determined. Mean ± SD. Protein expression was confirmed by immunoblotting.

episome that is organized in a compact chromatin conformation, with limited transcriptionally active regions within the viral genome (30–33). During reactivation, condensed viral chromosome gradually transitions into euchromatin state. Heterochromatin is characterized by trimethylation of H3K9 and assembly with HP1, which interacts with SUV39H1, a histone methylase (3). For KSHV, the viral latency protein LANA directly interacts with HP1 and links the latent viral episome to heterochromatin domains of the host cell (34–36). Beyond involvement of LANA and HP1, little is known about cellular factors involved in assembly of heterochromatin during KSHV latency nor the disassembly during reactivation. Here, we provide the first evidence that KAP-1 is involved in regulating KSHV latency. We showed that knockdown of KAP-1 significantly increases KSHV replication on K-Rta induction. Even in the absence of induction, knockdown of KAP-1 increased spontaneous viral reactivation in Vero-rKSHV.219 cells, which contain latent viral episomes.

KAP-1 has emerged as an important cellular factor in heterochromatin assembly. KAP-1 is a SUMO ligase (12), and when itself sumoylated, it interacts with SETBD1, a H3K9 histone methylase (3). KAP-1 also recruits histone deacetylases to remove acetyl residues from H3K9 and interacts with HP1 through its chromoshadow domain (37). One proposed model is that KAP-1 serves as an "enforcer" for heterochromatin formation by promoting H3K9m3 histone code through association with histone deacetylase and SETDB1, thus stabilizing HP1 and chromatin interactions. HP1, in concert with KAP-1, spreads heterochromatin via the associated SUV39H1 histone methylase. Disruption of the interaction between KAP-1 and HP1 converts the heterochromatin-like conformation to an open form (28). Here, we show that, in naturally infected BCBL-1 cells, KAP-1 occupancy on viral promoters was significantly higher during latency than in the lytic phase. To confirm that loss of KAP-1 binding was not due to encapsidation of new lytic viral genomes, we analyzed the expression profile of the virion protein K8.1 in KAP-1 knockdown cells. K8.1 was expressed at 24 h after KAP-1 dissociated from the viral genome, indicating that loss of KAP-1 binding happened before virus encapsidation (Fig. 1*C*). Consistently, decrease of KAP-1 occupancy during reactivation may dissociate HP1 from chromatin, resulting in demethylation of H3K9. Our data suggest that KAP-1 may regulate chromatin assembly and disassembly and thereby control viral latency. However, the present study does not address whether KAP-1 is the initiating factor or merely a facilitator of viral latency. KAP-1 has not previously been implicated in regulation of herpesvirus latency, although this cellular protein has been found to be associated with OriLyt (lytic replication origin) of EBV (38). KAP-1 is also involved in suppression of human papillomavirus transcription and replication mediated by viral gene E8-E2C (39) as well as silencing of murine leukemia virus proviral

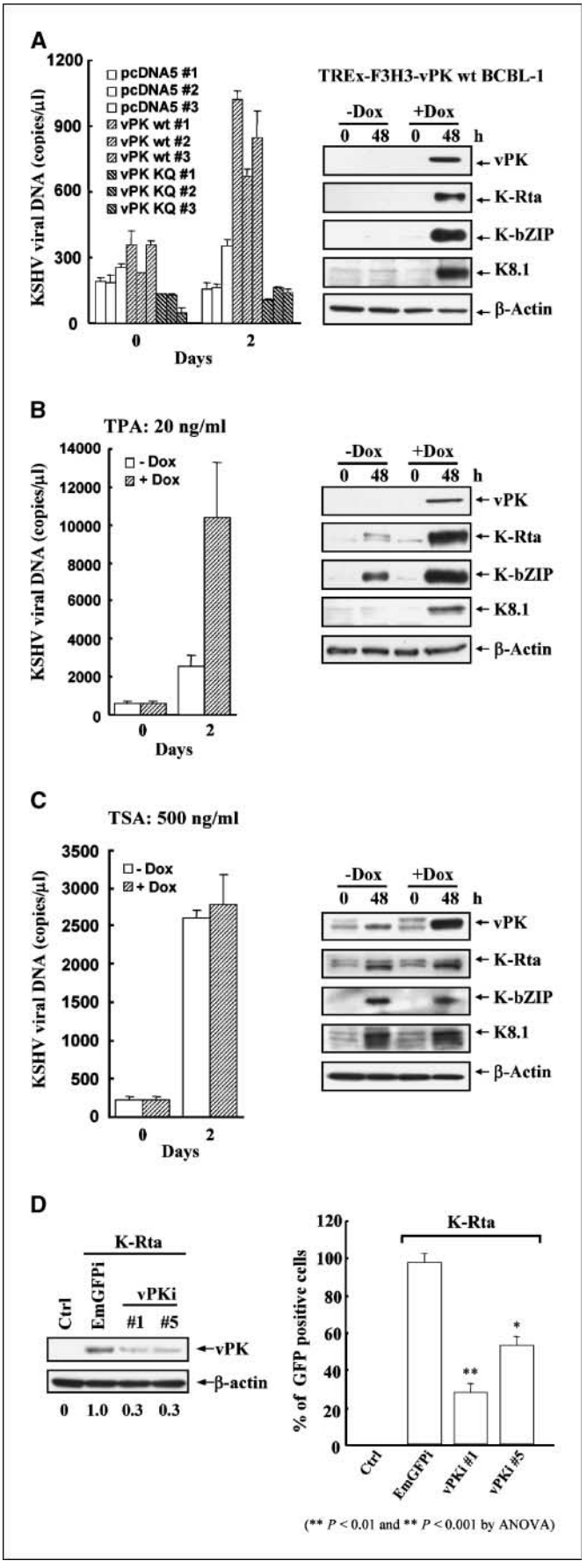


Figure 6. vPK enhances KSHV viral replication and lytic gene expression. *A,* supernatants of noninduced and doxycycline-induced TREx-F3H3-vPK BCBL-1 cells were collected and determined as described in Fig. 1*A* (*left*). Total cell lysates from the same cells were immunoblotted with the indicated antibodies (*right*). *B* and *C,* noninduced and doxycycline-induced TREx-F3H3-vPK BCBL-1 cells were incubated with TPA or TSA and subjected to detection of virion-associated KSHV DNA by quantitative PCR amplification. Total cell lysates from the same cells were immunoblotted with specific antibodies. *D,* Vero-rKSHV.219 cells were cotransfected with two different vPK shRNA constructs and K-Rta. Eighteen hours post-transfection, sodium butyrate (1 mmol/L) was applied to stimulate KSHV reactivation of K-Rta–treated cells. Forty-eight hours after transfection, total cell lysates were immunoblotted with anti-vPK and β-actin antibody. Filtered supernatants were collected at 72 h post-transfection and used to infect 293T cells. GFP-positive cells were counted by flow cytometry at 48 h post-infection. Data were normalized to % GFP-positive cells transfected with shEmGFP.

DNA in embryonic cells (40). Thus, it appears that multiple viruses exploit the KAP-1 pathway to silence genes and maintain a state of latency.

As a corepressor of Kruppel-associated box domain-containing zinc finger protein transcriptional factors, KAP-1 is associated with a wide spectrum of chromosomal sites (27). Currently, only a few target DNA consensus sequences have been identified in the Kruppel-associated box domain-containing zinc finger protein family; these targets include ZBRK1, ZNF202, and KS1. Based on sequence alignments, we found several ZBRK1 and ZNF202 binding sites within our viral USR library (Fig. 2*A, asterisk*). Thus, it is likely that KAP-1 associates with the KSHV genome via interaction with the ZBRK1 and ZNF202 transcriptional factors as well as other yet-to-be-determined transcriptional factors. A more comprehensive examination of the involvement of ZBRK1 and ZNF202 sites in KAP-1 binding awaits the development of high-avidity antibodies to these proteins.

We note that the ability of KAP-1 to condense chromatin is regulated by both sumoylation and phosphorylation (9, 10, 12, 13). Interaction with the histone methylase SETDB1 is critical for KAP-1 to compact chromosome, and this interaction depends on KAP-1 sumoylation (12). The autosumoylation ability of KAP-1 depends on the extent of phosphorylation. On DNA damage, KAP-1(Ser^{824}) phosphorylation by ATM decreases KAP-1 sumoylation and opens chromatin near DNA damage sites (11). KAP-1(Ser^{824}) phosphorylation also decreases the ability to repress gene expression (9). These reports prompted us to determine whether phosphorylation of KAP-1 by vPK played a role in the regulation of viral latency. We found that viral reactivation was accompanied by increased KAP-1(Ser^{824}) phosphorylation dependent on vPK. These studies showed that this phosphorylation by vPK interferes with sumoylation of KAP-1. We and others have shown previously that vPK is an early gene product and packaged into the virion (24, 41). The expression pattern of vPK correlates with the appearance of phospho-KAP-1(Ser^{824}), and vPK levels affect KSHV reactivation. We also showed that vPK can further enhance chemically induced reactivation by TPA but not TSA. These data, when taken together, support a model whereby vPK regulates viral latency by phosphorylating KAP-1 to ensure that the KAP-1 heterochromatin complex remains dissociated from the viral episome. It should be noted that vPK may not be absolutely required for KSHV replication under all conditions and in all cell types. EBV vPK (BGLF4) was shown to be required for viral replication (42); however, vPK/ORF36 of murine γ-herpesvirus 68 is critical for infection of primary macrophages but not fibroblasts (43). Our current study also showed that vPK phosphorylated the same site of KAP-1 as ATM. Tarakanova and colleagues (43) reported that the viral kinases of murine γ-herpesvirus 68 and EBV both phosphorylate γH2AX, a substrate of ATM during DNA damage; these findings revealed overlapping phosphorylation specificity of vPK and certain cellular protein kinases. We also found that KSHV vPK phosphorylated γH2AX *in vitro* (data not shown). Thus, it is conceivable that vPK may play a diminished role in viral replication under conditions where ATM activity is high. In this regard, it is interesting that Shin and colleagues reported that the KSHV vIRF protein interacts with and attenuates ATM activity in infected cells (44), likely accentuating the importance of vPK in KSHV replication.

Using mass spectroscopy, we found that KAP-1 is a major cellular protein associated with vPK. This was confirmed in cells overexpressing vPK as well as in naturally infected BCBL-1 cells. Taking advantage of the phosphorylation site mutant of KAP-1, we found that vPK contributes to the phosphorylation at Ser^{824}. However, KAP-1-S824A remained slightly phosphorylated, suggesting that there are other vPK phosphorylation sites on KAP-1, which have yet to be mapped and characterized. Other than KAP-1, the predominant cellular proteins that interact with vPK are mostly chaperone proteins (heat shock protein 70, heat shock protein 90, cdc37, etc.; ref. 45).

In summary, the present study provides new findings on the regulation of KSHV replication by analysis of viral and cellular proteins that control transcription by influencing chromatin dynamics. First, KAP-1 was shown to be a regulator of viral latency, and its association with transcriptional promoters in the viral genome correlated with the formation of heterochromatin complexes. Second, KAP-1 was modulated by vPK phosphorylation; this post-translational modification prevents KAP-1 from associating with chromatin, ensuring a fully relaxed viral chromatin state conducive to lytic replication.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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