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Kaposi's Sarcoma-Associated Herpesvirus Viral Protein Kinase Interacts with RNA Helicase A and Regulates Host Gene Expression

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RNA helicase A (RHA) containing the DExH motif is a human homolog of maleless protein that regulates expression of genes located in the *Drosophila* X chromosome during dosage compensation. RHA exerts helicase activity that unwinds double-stranded RNA and DNA to a single-strand form. The protein acts as a bridging factor mediating interactions of CBP/p300 and RNA pol II, and consequently affects gene expression. Kaposi's sarcoma-associated herpesvirus (KSHV) is a member of the γ-herpesvirus subfamily that causes several disorders. The majority of herpesviruses commonly encode predicted viral protein kinases. KSHV open reading frame 36 (ORF36) codes for protein kinase domains, and functions as a serine/threonine protein kinase. KSHV ORF36 is classified as a late gene, as it is expressed during lytic replication and localized in the nuclei of KSHV-infected cells. Recent studies show that viral protein kinase (vPK) interacts with cellular proteins. In this study, we determined the cellular localization of vPK in KSHV-infected BCBL-1 cells using confocal microscopy. Proteomic analysis indicates that cellular proteins interacted with vPK, and co-immunoprecipitation reactions further reveal interactions between vPK and RHA. Moreover, KSHV vPK appeared to regulate the transcriptional activation of Cre promoter, and plays an important role in cellular transcription of RHA.

***Keywords*:** Kaposi's sarcoma-associated herpesvirus, viral protein kinase, RNA helicase A, CREB-binding protein

Kaposi's sarcoma-associated herpesvirus (KSHV), also termed human herpesvirus 8, was distinguished from Kaposi's sarcoma (KS) in patients with acquired immunodeficiency syndrome (AIDS) in 1994 (Chang *et al.*, 1994). KSHV causes a number of diseases, such as Primary Effusion Lymphoma (PEL), Multicentric Castleman disease (MCD), and KS (Cesarman *et al.*, 1995; Soulier *et al.*, 1995). Based on genomic structures and biological properties, herpesviruses are divided into α, β, and γ subfamilies (Roizman *et al.*, 1981). KSHV classified into the γ-herpesvirus subfamily based on sequence analysis, and is very closely related to Epstein-Barr virus (EBV) (Russo *et al.*, 1996). Most KSHV genes encode viral proteins homologous to cellular proteins involved in cell proliferation and tumorigenesis, such as interleukin-6, G-protein coupled receptor, and interferon regulatory factor (Russo *et al*., 1996; Arvanitakis *et al.*, 1997; Neipel *et al.*, 1997; Cathomas, 2003). Similar to other herpesviruses, KSHV operates via both latent and lytic cycles. During the latent cycle, only limited genes are expressed, including latency-associated nuclear antigen 1 (Parravicini *et al.*, 2000), and the viral genome exists in a circular form (an episome) (Decker *et al.*, 1996; Zhong *et al.*, 1996). In contrast, during the lytic cycle, viral genes required for assembly of the virion and emission are expressed, leading to destruction of host cells and synthesis of virus particles (Deng *et al.*, 2007). Rta encoded by KSHV open reading frame 50 (ORF50) is necessary and sufficient to reactivate KSHV from the latent to lytic cycle in infected cells (Lukac *et al.*, 1998). Reactivation of KSHV by Rta can be induced by chemical agents, such as 12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA) and sodium butyrate (Yu *et al.*, 1999).

The majority of herpesviruses commonly encode predicted viral protein kinases (vPKs). The vPK proteins of the herpesvirus family contain 11 conserved domains and share serine/threonine protein kinase motifs (Smith and Smith, 1989; Kawaguchi and Kato, 2003). Earlier studies reported that vPK mimics the functions of cellular protein kinases, such as Cdc2 and EF-δ (Kawaguchi and Kato, 2003). Mutagenesis experiments show that the lysine residue located in subdomain II is essential for catalytic activity. The phosphorylation of proteins by herpesvirus is important for viral survival, replication, and pathogenesis. Herpes simplex virus US3, varicella-zoster virus ORF66, Epstein-Barr virus BGLF4, and Herpesvirus saimiri ORF36 have been identified as vPK proteins. KSHV vPK encoded by ORF36 also contains protein kinase domains, and functions as a serine/threonine protein kinase. KSHV vPK is a late gene expressed during lytic replication that localizes in cell nuclei (Park *et al.*, 2000; Izumiya *et al.*, 2007; Park *et al.*, 2007). The protein is autophosphorylated via a lysine residue within subdomain II, similar to the vPK proteins of other herpesviruses (Park *et al*., 2000). Recent studies showed that vPK interacts with and phosphorylates cellular proteins involved in the c-Jun N-terminal Kinase (JNK) pathway, activates this signal transduction pathway, and facilitates viral replication (Hamza *et al.*, 2004). In addition, KSHV vPK binds Focal Adhesion Kinase (FAK) and inhibits FAK activity, leading to transformation of cell morphology to a round shape

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(Park *et al*., 2007).

The DExD/H family contains several highly conserved proteins (Linder *et al.*, 1989). Recently, several investigators have expressed considerable interest in proteins of this family, because they play various important roles, in association with cellular proteins, in RNA metabolic processes (Tanner and Linder, 2001; Fuller-Pace, 2006). RNA helicase A (RHA) contains the DExH motif, and is a human homolog of maleless proteins that regulate expression of genes located in the *Drosophila* X chromosome during dosage compensation (Lee and Hurwitz, 1993). RHA exerts helicase activity that unwinds double-stranded RNA and DNA to the single-stranded form (Lee and Hurwitz, 1992). CREB binding protein (CBP/p300), a co-activator with histone acetyltransferase activity (Bannister and Kouzarides, 1996), is linked to the transcription of a variety of genes. Previous studies showed that RHA interacts with CBP/p300 and regulates transcription. RHA additionally interacts with RNA polymerase II (RNA pol II). RHA has been identified as a bridging factor that mediates interactions of CBP/p300 with RNA pol II. Mutants of RHA suppress transcription activity involved in CBP/p300 (Nakajima *et al.*, 1997a). Moreover, RHA mediates a similar association between Breast cancer-specific tumor suppressor protein 1 (BRCA1) and RNA pol II, and promotes BRCA1 activity as a transcription factor (Anderson *et al.*, 1998). BRCA1 performs several functions, including transcriptional regulation of genes involved in DNA repair, apoptosis, the cell cycle, chromatin remodeling, and X chromosome inactivation (Mullan *et al.*, 2006). RHA additionally interacts with retroviral elements and stimulates viral gene expression (Chang *et al*., 1994; Reddy *et al.*, 2000; Tang and Wong-Staal, 2000). Accordingly, the expression levels of a number of genes are altered, depending on the presence of RHA. RHA plays an important role in regulating transcription. Discovery and understanding of the mechanisms of interaction between vPK and cellular proteins should allow clarification of the cellular functions of vPK. We conducted experiments to identify the cellular proteins interacting with KSHV vPK, with a view to determining the function of the viral protein. Our data support interactions between vPK and RHA. Based on the results, we propose that the transcription activity of RHA is regulated by KSHV vPK.

Materials and Methods

Plasmids and antibodies

GST-vPK was generated, as described previously (Park *et al*., 2000). The GST-vPK (K108A) mutant was constructed using the Quik-Change^{R} Site-Directed Mutagenesis kit (Stratagene, USA). Deletion mutants of GST-vPK were created using PCR, and subcloned into the pEBG vector. pEBG had been digested with *Bam*HI/*Not*I and PCR-amplified DNA had been partially digested with *Bam*HI/*Not*I. HA-RHA expression plasmid and anti-RHA antibody were kind gifts from Dr. Jeffrey Parvin (Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA). The pEGFP-C1 plasmid was employed as a GFP expression vector. vPK and K8 polyclonal antibodies were produced in rabbits.

Cell culture and transfection

293T cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. BCBL-1 cells were maintained in RPMI 1640 containing 10% fetal bovine serum. BCBL-1 cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA). Transfections were performed using the calcium phosphate precipitation method or Fugene-6 reagents (Roche, Germany), according to the manufacturer's recommendations.

In vivo GST pull down assay

293T cells were transiently co-transfected with GST or GST-vPK. After 48 h transfection, cells were lysed with EBC buffer (50 mM Tris-HCl; pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride), and incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, UK) at 4°C for 2 h with rocking. Bound protein complexes were washed three times with EBC buffer, and heated to 95°C for 5 min in SDS sample buffer. Bound proteins were analyzed by SDS-PAGE and identified using mass spectrometry.

In vivo binding assays

293T cells were transiently co-transfected with GST or GST-vPK in combination with HA-RHA. After 48 h, cells were lysed with EBC buffer and incubated with glutathione-Sepharose 4B (GS4B) beads at 4°C for 2 h with rocking. Bound protein complexes were washed three times with EBC buffer, and heated to 95°C for 5 min in SDS sample buffer. Western blots were performed using anti-HA and anti-GST mouse monoclonal antibodies. TPA-induced-BCBL-1 cells were lysed with EBC buffer and immunoprecipitated with anti-HA, anti-vPK, and anti-K8 antibodies. Samples were immunoblotted with the anti-RHA antibody.

Immunofluorescence

Cells were fixed with 3.7% formaldehyde for 30 min, permeabilized using phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (PBST) on ice, and blocked with 1% bovine serum albumin (BSA). Next, cells were incubated with anti-HA antibody in PBST containing 1% BSA for 1 h at room temperature and washed with PBST, followed by treatment with TRITC-conjugated anti-mouse antibody in PBST containing 1% BSA for 1 h and further washing with PBST. Cells were analyzed using a Zeiss (Germany) confocal microscope with fluorescein isothiocyanate (FITC).

Reporter assays

At 24 h after transfection, cells were harvested and equal amounts of extracts employed for the detection of luciferase activity measured using a luminometer. The total amount of transfected DNA was maintained by the addition of blank vector. Activity of the reporter alone was set at a value of 1, and luciferase measurements normalized to those of β-galactosidase employed as the internal control.

Results

Cellular localization of vPK in KSHV-infected BCBL-1 cells

Previous studies showed that vPK is localized in the nuclei of transiently transfected cells (Park *et al*., 2000, 2007; Izumiya *et al.*, 2007). Experiments were performed to ascertain the subcellular location of vPK in KSHV-infected BCBL-1 cells during the lytic cycle. We treated BCBL-1 cells with TPA to induce reactivation, because vPK is expressed during the lytic cycle. After 48 h of reactivation with TPA, we performed an immunofluorescence assay using vPK-specific antibodies and

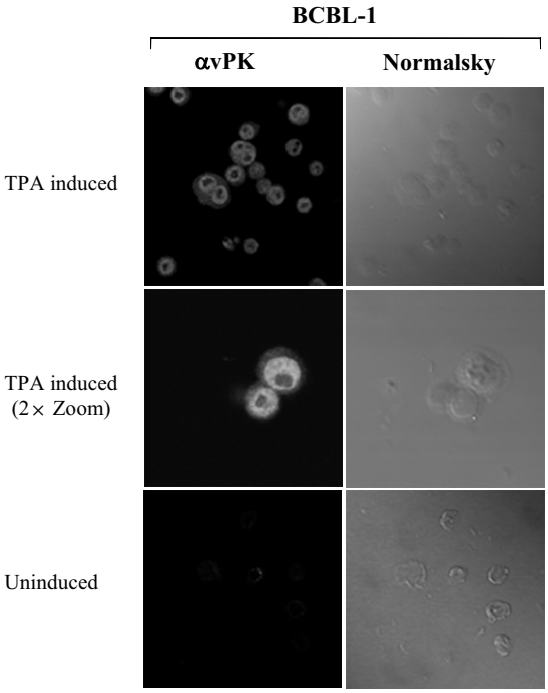

Fig. 1. Cellular location of vPK in KSHV-infected BCBL-1 cells. Analyses were performed using a Zeiss confocal microscope with fluorescein isothiocyanate (FITC). We observed cells 48 h after treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) (20 ng/ml). vPK (green) was detected in the nuclei of infected cells treated with TPA.

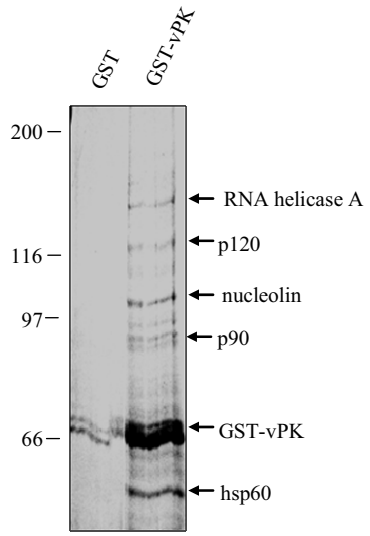

Fig. 2. Identification of cellular proteins interacting with KSHV vPK. 293T cells were transfected with GST empty vector (control) or GST-vPK, and harvested 48 after transfection. Cell extracts were precipitated with GS4B beads and loaded on SDS-polyacrylamide gels. After destaining, proteins were identified using mass spectrometric analysis.

examined BCBL-1 cells using confocal microscopy. vPK was detected in the nuclei of infected cells treated with TPA (Fig. 1, upper and middle panels). In contrast, no expression of vPK was seen in control BCBL-1 cells (Fig. 1, bottom panels).

Identification of cellular proteins interacting with KSHV vPK

To identify cellular proteins that bind vPK, experiments were conducted using Glutathione-S-transferase (GST)-vPK fusion proteins. 293T cells were transfected with GST or GST-vPK vectors and harvested after 48 h. Prepared cell extracts were precipitated using GS4B beads, and separated by SDS-PAGE. We did not detect any cellular proteins interacting with GST (except for a non-specific band), whereas several proteins interacted with GST-vPK (Fig. 2 right). Mass spectrometry of proteins from the gel led to the identification of RHA, nucleolin, heat shock protein 60, and unknown proteins of 90 kDa and 120 kDa (Fig. 2). Subsequent experiments focused on RHA.

KSHV vPK interacts with RHA

To determine whether vPK interacts with RHA in 293T cells, co-immunoprecipitation (co-IP) analysis was performed *in vivo*. Extracts from 293T cells transfected with GST or GST-vPK were precipitated with GS4B beads, followed by immune-blotting using RHA-specific antibodies. RHA was not detected in 293T cells transfected with GST (Fig. 3A, lane 3), whereas interactions between vPK and RHA were evident in 293T cells expressing vPK (Fig. 3A, lane 4). To confirm interactions between endogenous vPK and RHA, KSHV-infected BCBL-1 cells were subjected to immunoprecipitation analysis. As mentioned earlier, lytic reactivation was induced in BCBL-1 cells by TPA, and immunoprecipitates were subjected to western blotting with RHA antibodies to detect protein. RHA was present in anti-vPK precipitates, but not upon immunoprecipitation of extracts with anti-K8 or anti-HA antibodies. Our findings clearly indicate that endogenous vPK interacts with RHA.

vPK N-terminal regions are required for RHA interactions

To identify the specific regions of vPK required for interactions with RHA, we performed GST pulldown assays using vPK deletion mutants. One mutant contained the N-terminal region (aa 1-83), whereas another contained the protein kinase domain and the C-terminal region (aa 84-444) (Fig. 4A). Mutants of vPK were generated using PCR, and subcloned into pEBG vector. 293T cells were co-transfected with GST-vPK mutants and HA-RHA, and harvested at 48 h after transfection. We performed immunoprecipitation analysis with GS4B beads and Western blotting with anti-HA antibodies. RHA interacted specifically with the N-terminal region of vPK (Fig. 4B, GST-precipitated lane 2), but did not interact with GST or the other vPK mutant (aa 84-444). Accordingly, we propose that the N-terminal region (aa 1-83) of vPK is crucial for RHA interaction.

vPK co-localizes with RHA

RHA is predominantly localized in the nucleus, but shuttles between the nucleus and cytoplasm. Thus, RHA is dynamic (Smith *et al.*, 2004; Fujita *et al.*, 2005; Aratani *et al.*, 2006; Liu *et al.*, 2007). We co-transfected 293T cells with HA-RHA and

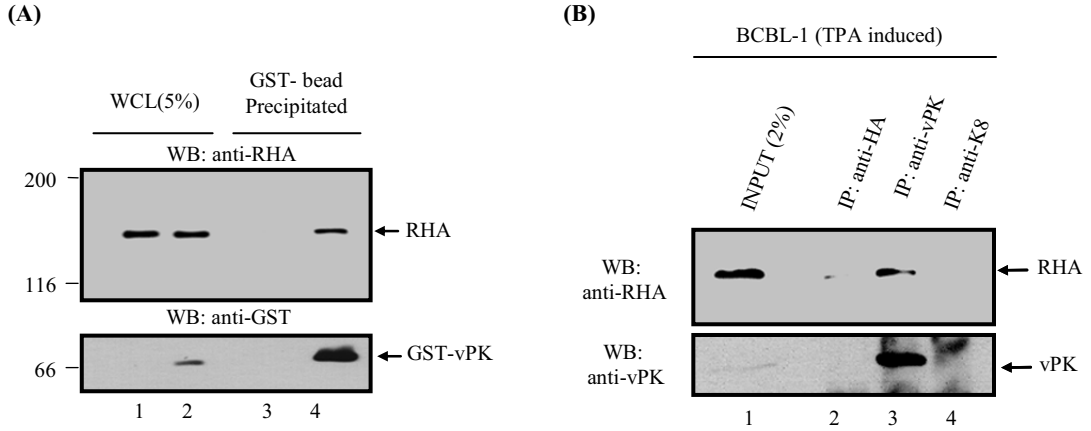

Fig. 3. Interaction between vPK and RHA. (A) Interactions between vPK and RHA in 293T cells cotransfected with GST-vPK and RHA. At 48 h after transfection, 293T cells were lysed with EBC buffer. Cell extracts were precipitated with GS4B beads, and subjected to Western blot analysis with RHA-specific antibodies. RHA was specifically detected in extracts of 293T cells transfected with GST-vPK. Lanes: 1 and 3, 293T cells transfected with GST only; 2 and 4, 293T cells transfected with GST-vKP. (B) Identification of interactions between vPK and RHA in KSHV-infected BCBL-1 cells induced with TPA. At 48 h after transfection, cells were harvested. Cell extracts were immunoprecipitated with vPK-specific antibodies and subjected to Western blotting using RHA-specific antibodies. Anti-K8 and HA antibodies were used as controls.

GFP-vPK, or GFP alone, and determined the subcellular locations of the two proteins using confocal microscopy. We expected to see co-localization, as the two proteins interact within cells. Interestingly, RHA was detected in the nuclei of 293T cells co-transfected with GFP alone and HA-RHA, but did not coincide with the position of GFP. In contrast, both vPK and RHA were identified in the nuclei of 293T cells co-transfected with GFP-vPK and HA-RHA (Fig. 5).

vPK inhibits the activation of Cre promoter

CBP/p300 is a co-activator that stimulates CREB-dependent transcription by associating with specific transcription factors. However, CBP must interact with RNA pol II to achieve transactivation, and this interaction is mediated by RHA. Thus, RHA stimulates the transcriptional activity of CBP. To establish whether vPK-RHA interactions affected transcription by CBP/p300, we measured transcription activity using a Cre promoter-Luciferase reporter gene (Cre-Luc). 293T cells were co-transfected with protein kinase A (PKA), vPK, and Cre-luc, harvested, and luciferase activity was measured with a luminometer. Transcription activity of the Cre promoter in the presence of PKA fell with increasing doses of vPK (Fig. 6A). In contrast, the vPK deletion mutants did not suppress transcriptional activation of the Cre promoter by CBP/p300. Interestingly, the vPK kinase-dead mutant (K108A) did not inhibit promoter activity (Fig. 6B). The result suggests that the binding to vPK is insufficient for the inhibition of Cre promoter. Based on these results, we suggest that KSHV vPK inhibits PKA-induced Cre promoter activation and the protein kinase function of vPK is required for inhibition.

Fig. 4. The regions of vPK required for vPK-RHA interactions. (A) vPK deletion mutants were designed using PCR. One mutant contained aa 1-88, while the other included aa 84-444. (B) 293T cells were co-transfected with HA-RHA and GST or GST-vPK mutants. At 48 h after transfection, 293T cells were harvested and lysed with EBC buffer. Cell extracts were precipitated with GS4B beads, and subjected to Western blotting with HA antibodies.

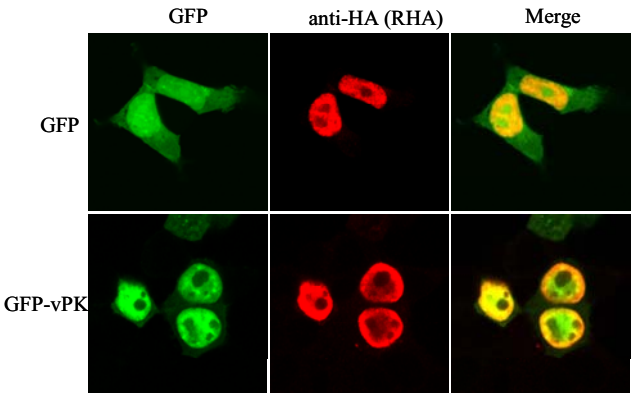

Fig. 5. Co-localization of vPK and RHA. 293T cells were co-transfected with HA-RHA and GFP or GFP-vPK. Proteins were detected using confocal microscopy. GFP or GFP-vPK subcellular location is presented in green, while RHA is red. The yellow color indicates co-localization.

Discussion

KSHV, a member of the γ-herpesvirus subfamily, is linked to several malignancies in humans, including KS, PEL, and MCD (Cesarman *et al*., 1995; Soulier *et al*., 1995). KSHV displays both latent and lytic phases of infection. KSHV ORF36 encodes a serine protein kinase expressed during the viral lytic cycle. Our results support previous data showing that vPK is localized in the nuclei of transiently transfected and KSHV-infected cells (Fig. 1) (Park *et al.*, 2000, 2007; Izumiya *et al*., 2007). Proteins encoded in the KSHV genome affect signal transduction in host cells, and are involved in the expression of both viral and host genes. Recent studies have demonstrated that vPK binds to and phosphorylates specific proteins, resulting in activation of the JNK pathway (Hamza *et al*., 2004). To establish the physiological roles of vPK, we identified the interacting cellular proteins, including RHA, using proteomics analysis (Fig. 2). RHA acts as a helicase to unwind dsRNA and dsDNA in the 3′- to -5′ direction, and displays transactivation activity (Lee and Hurwitz, 1992).

CREB, a transcription factor, is activated by various signal pathways. Receptor stimulation results in activation of PKA, one of the upstream proteins that enhance CREB activity. Subsequently, the catalytic subunit of PKA translocates to the nucleus and phosphorylates CREB, which binds to the Cre promoter, resulting in expression of target genes (Della Fazia *et al.*, 1997; Shaywitz and Greenberg, 1999). Phosphorylated CREB additionally associates with a CBP/p300 coactivator to stimulate expression of genes controlled by the Cre promoter. CBP/p300 connects CREB with the basal transcriptional machinery (Nakajima *et al.*, 1997b). Interaction between CBP/p300 and RNA poll II requires RHA. RHA binds both CBP/p300 and RNA pol II, and acts as a bridging factor that mediates interactions between CBP/p300 and RNA pol II, thus stimulating the transcriptional activity of CBP (Nakajima *et al*., 1997a; Zhang and Grosse, 2004; Fuller-Pace, 2006). Transient reporter assay using RNA helicase-defective mutant of RHA (K417R) revealed that the helicase activity of RHA has an effect on Cre promoter activation. RHA may improve transcriptional activity by change in chromatin structure from dsDNA to ssDNA (Nakajima *et al*., 1997a). Previous study demonstrated that RHA mutants lacking ATP binding activity showed significantly decreased transcriptional activity compared with wild type (wt) RHA. In addition, wt RHA enhanced CREB dependent transcription in wt PKA transfected cells

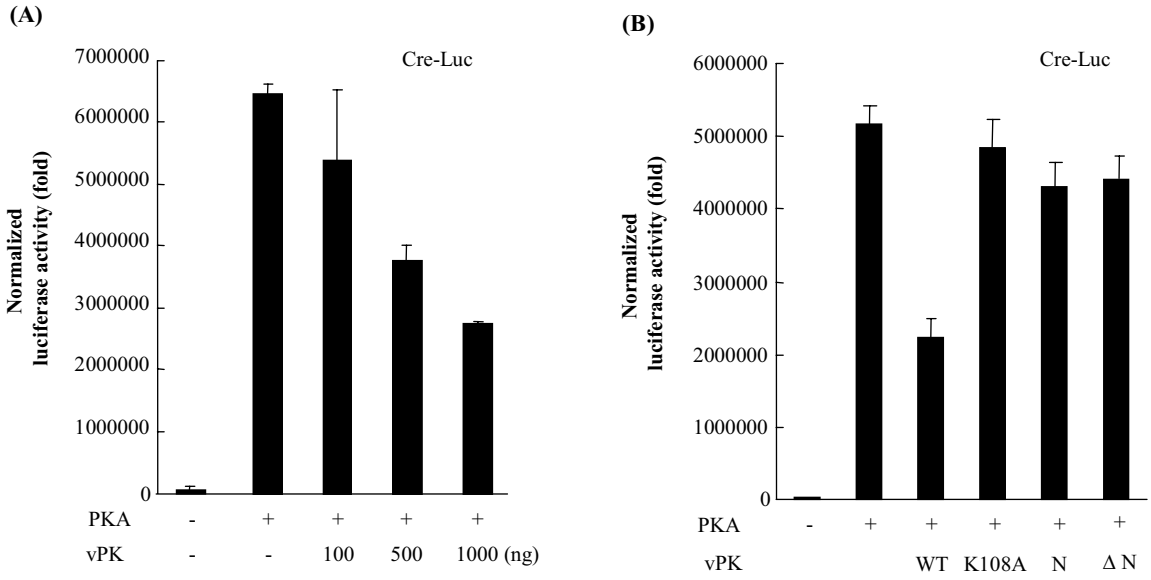

Fig. 6. Regulation of transactivation of RHA by vPK. (A) 293T cells were co-transfected with PKA and Cre-Luc and vPK. At 24 h after transfection, cells were harvested, and luciferase activity measured using a Luminometer. Experiments were performed in triplicate. Each value represents the mean of 3 experiments±standard deviations. (B) 293T cells were co-transfected with PKA, Cre-Luc and vPK mutants (K108A; N, N-terminal region; ΔN, N-terminal region deletion mutants).

but not in mutant PKA transfected cells (Aratani *et al.*, 2001). These data show that RHA involved in PKA-induced Cre promoter activation and helicase activity contributes to transactivation ability of RHA. According to previous studies, RHA binds to the Ku antigen, which is the DNA binding subunit of DNA-dependent protein kinase (DNA-PK), and is phosphorylated by DNA-PK. Phosphorylation of RHA depends on RNA and DNA, indicating that it may play important roles in RNA and DNA metabolisms (Zhang and Grosse, 1994; Zhang *et al.*, 2004). DNA-PK–catalyzed phosphorylation of the RHA is necessary and sufficient to the expression of P-glycoprotein encoded by *MDR1* gene in multidrug-resistant human leukemia cells (Zhong and Safa, 2007). Our research shows that RHA interacts with vPK and that its transactivation ability could be controlled by vPK. Co-IP analysis disclosed interactions of RHA with KSHV vPK *in vivo* (Fig. 3A). We performed experiments showing that endogenous vPK binds RHA in KSHV-infected BCBL-1, and identified the specific interactions between the two proteins (Fig. 3B). RHA is predominantly localized in the nucleus, but shuttles from the nucleus to the cytoplasm (Smith *et al*., 2004; Fujita *et al*., 2005; Aratani *et al*., 2006; Liu *et al*., 2007). Co-localization of vPK and RHA was observed (Fig. 5). We performed experiments using a Cre-Luc reporter gene to determine how interactions between vPK and RHA affect the transactivation ability of RHA in CREB-dependent transcription regulation. Transcriptional activity of the Cre promoter, stimulated by PKA, fell in a dose-dependent manner in 293T cells co-transfected with vPK (Fig. 6A), but was not affected by a vPK kinase-dead mutant (K108A) (Fig. 6B). In summary, KSHV ORF36 expressed during the lytic cycle interacts with RHA and regulates its transactivation.

We further determined the vPK region required for RHA interactions, using deletion mutants. Our team is currently focusing on mapping of the RHA region required for interacttions with vPK. We aim to establish whether vPK competently binds to the RHA regions interacting with CBP/p300. Experiments that confirm direct interactions between vPK and RHA, including GST-pull down analysis with *in vitro* translated RHA, are required. Future studies should aim to determine whether RHA is phosphorylated by vPK and the mechanisms underlying inhibition of the transactivation ability of RHA. RHA additionally mediates association of BRCA1 and RNA pol II (Anderson *et al*., 1998). Analysis of the influence of vPK-RHA interacttions on gene expression stimulated by BRCA1 will be possible, using the techniques described above.

Herpesviruses commonly encode vPK, and perform known functions, including viral replication, regulation of host genes, and signal transduction. A recent study showed that interferon sensitivity was increased when vPK encoded by murine γ-herpesvirus 68 (MHV-68, similar to KSHV) was removed (Hwang *et al.*, 2009). The results support the possibility of treating KSHV-infected cells by inhibiting vPK activity using interferon. KSHV vPK may play important roles in virus survival and development of disease. Further studies are needed to establish the physiological roles of vPK during lytic function.

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