Screening of the Human Kinome Identifies MSK1/2-CREB1 as an Essential Pathway Mediating Kaposi’s Sarcoma-Associated Herpesvirus Lytic Replication during Primary Infection

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ABSTRACT

Viruses often hijack cellular pathways to facilitate infection and replication. Kaposi’s sarcoma-associated herpesvirus (KSHV) is an oncogenic gammaherpesvirus etiologically associated with Kaposi’s sarcoma, a vascular tumor of endothelial cells. Despite intensive studies, cellular pathways mediating KSHV infection and replication are still not well defined. Using an antibody array approach, we examined cellular proteins phosphorylated during primary KSHV infection of primary human umbilical vein endothelial cells. Enrichment analysis identified integrin/mitogen-activated protein kinase (integrin/MAPK), insulin/epidermal growth factor receptor (insulin/EGFR), and JAK/STAT as the activated networks during primary KSHV infection. The transcriptional factor CREB1 (cyclic AMP [cAMP]-responsive element-binding protein 1) had the strongest increase in phosphorylation. While knockdown of CREB1 had no effect on KSHV entry and trafficking, it drastically reduced the expression of lytic transcripts and proteins and the production of infectious virions. Chemical activation of CREB1 significantly enhanced viral lytic replication. In contrast, CREB1 neither influenced the expression of the latent gene LANA nor affected KSHV infectivity. Mechanistically, CREB1 was not activated through the classic cAMP/protein kinase A (cAMP/PKA) pathway or via the AKT, MK2, and RSK pathways. Rather, CREB1 was activated by the mitogen- and stress-activated protein kinases 1 and 2 (MSK1/2). Consequently, chemical inhibition or knockdown of MSKs significantly inhibited the KSHV lytic replication program; however, it had a minimal effect on LANA expression and KSHV infectivity. Together, these results identify the MSK1/2-CREB1 proteins as novel essential effectors of KSHV lytic replication during primary infection. The differential effect of the MSK1/2-CREB1 pathway on the expression of viral latent and lytic genes might control the robustness of viral lytic replication, and therefore the KSHV replication program, during primary infection.

IMPORTANCE

Kaposi’s sarcoma-associated herpesvirus (KSHV) is a human tumor virus associated with several cancers. Through genome-wide kinase screening, we found that KSHV activates the MSK1/2-CREB1 pathway during primary infection and that it depends on this pathway for viral lytic replication. Inhibition of this pathway blocks KSHV lytic replication. These results illustrate a mechanism by which KSHV hijacks a cellular pathway for its replication, and they identify a potential therapeutic target.

Successful KSHV infection requires the coordinated expression and trafficking of viral gene products, leading to the activation of signal transduction pathways. A number of these pathways regulate KSHV entry, trafficking, and viral gene expression. Binding of KSHV glycoproteins to cellular receptors activates focal adhesion kinase (FAK), Src, phosphatidylinositol-3-kinases (PI3Ks), and mitogen-activated protein kinases (MAPks), including MEK/extracellular signal-regulated kinase (MEK/ERK), p38, and Jun N-terminal kinase (JNK), facilitating KSHV internalization and trafficking. This process depends on rearrangements of the actin and microtubule cytoskeletons and on factors, such as Rho-GTPases and diaphanous-2 (Dia-2), that regulate their dynamics. KSHV entry and trafficking rely on the dynamics of the ubiquitin/proteasome system and on activation of the E3 ligase c-Cbl to maintain the endosomal activities and cellular signaling.
sion of viral genes. Whether KSHV enters into latency or undergoes lytic replication depends on the extent of expression of viral lytic genes. Several signaling pathways, including ERK, p38, and JNK pathways, promote the expression of viral lytic genes, while the NF-κB pathway promotes the expression of viral latent genes (9, 14, 15). In permissive primary human umbilical vein endothelial cells (HUVEC), in which the ERK, p38, and JNK pathways are highly activated upon KSHV infection, the virus undergoes robust productive lytic replication before entering into latency (9, 16, 17). In contrast, in nonpermissive primary human dermal microvascular endothelial cells (DMVEC) and human foreskin fibroblasts (HFFs), KSHV enters a default latency program with minimal viral lytic activity, which parallels the hyperactivation of the NF-κB pathway (15, 18–20). The ERK, p38, and JNK pathways promote lytic replication by activating AP-1 complexes to induce the expression of RTA (Orf50), the master transactivator of KSHV lytic replication, and other viral lytic genes (9, 21). On the other hand, the NF-κB pathway inhibits viral lytic replication by suppressing the AP-1 complexes (22).

Despite these observations, whether KSHV hijacks any other cellular pathways to promote infection and replication during primary infection has not been examined systematically. The present study aimed to identify additional essential signaling pathways and kinases that regulate primary KSHV infection. To this end, we performed an antibody-based kinase screening assay. This assay uses 334 phospho-site-specific antibodies against 287 different phosphorylation sites in ~200 protein kinases, phosphatases, and other cell signaling proteins that regulate cell proliferation, stress, and apoptosis. Our screen identified 29 phospho-sites on 24 kinases or signaling molecules whose phosphorylation levels were significantly altered at early time points (<15 min) of primary KSHV infection. These hits included protein kinase B (AKT1) and c-Jun, whose activation and roles in primary KSHV infection were previously reported (9, 15). Other hits, such as protein kinase C-δ (PKCδ), were previously implicated in KSHV reactivation (23), though the role of PKCδ in primary KSHV infection has not been explored.

The top hit, CREB1 (cyclic AMP [cAMP]-responsive element binding protein 1), is a member of a subfamily of bZIP transcription factors that includes ATF1 and CREM. CREB1, through binding to a conserved DNA sequence called the cAMP-responsive element (CRE), acts downstream of a wide variety of signals in cells to mediate many functions, including immune function, memory, metabolism, and cell survival (24–28). CREB1 has been shown to regulate the expression of viral latent and lytic genes to determine the KSHV replication program, during primary infection.

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KSHV BAC16-infected iSLK (iSLK-BAC16) cells were maintained in the presence of 1 µg/ml puromycin, 250 µg/ml G418 (Sigma-Aldrich), and 1,200 µg/ml hygromycin B (46). Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1X penicillin-streptomycin solution (Genscience Scientific, San Diego, CA).

**Virus preparation.** A volume of concentrated virus was prepared from iSLK-BAC16 cells as previously described (46). Briefly, iSLK-BAC16 cells were induced with both doxycycline (1 µg/ml) and sodium butyrate (1 mM) in the medium described above, but without hygromycin, puromycin, and G418. Four days later, the supernatant was collected, centrifuged at 5,000 × g for 10 min, and then filtered (0.45-µm pore size) to eliminate cell debris. Virus particles were pelleted by ultracentrifugation (100,000 × g for 1 h with a 20% sucrose cushion at 4°C), using an SW32 Ti rotor (Beckman Coulter Inc., Brea, CA). The final pellet was dissolved in culture medium overnight and used for infection after titration.

**Virus titration.** A fresh virus preparation or culture supernatant was titrated by infecting HUVEC as previously described (16, 47). Briefly, the virus preparation or supernatant was subjected to 2-fold serial dilution and used to infect HUVEC at 2 × 10⁴ cells/well in a 24-well plate, at 20 µl/well, in the presence of Polybrene. Three to six infection repeats were carried out for each sample. Four hours following infection, the medium was replaced with fresh medium. The plates were examined with an inverted fluorescence microscope at 48 h postinfection to determine the number of cells expressing green fluorescent protein (GFP) in each well. Infectious units (IU) were calculated based on the number of GFP-positive cells generated with a 1-ml sample.

**Virus infection.** We conducted primary infection of HUVEC to examine factors that might regulate KSHV infection and replication. Fresh virus preparations with a titer of 2 × 10⁶ IU were used in the experiments. HUVEC were infected with KSHV as previously described (16, 47). For all experiments, cells were infected at a multiplicity of infection (MOI) of 2 IU per cell unless specified otherwise. To prepare a replication-defective virus, KSHV was exposed to a UV source for 15 min, which reduced virus infectivity from 70 to 80% to less than 1%. To determine the effects of kinase inhibitors or agonists on KSHV infection, HUVEC grown to confluence in 36-mm dishes were first serum-starved for 4 h and then treated with kinase inhibitors or agonists (LY294002, SB747651A, PF3644022, or FSK) for 1 h at 37°C prior to KSHV infection. Cells were then infected with KSHV in the presence of the inhibitors or agonists. Cells were collected at different time points, as specified, and used to examine the expression of viral transcripts and proteins. To examine the production of infectious virions, supernatants were collected at day 4 postinfection and subjected to titration as described in the previous section.

**Kinex antibody microarray and data analysis.** HUVEC that were serum-starved for 4 h were mock infected or infected with KSHV for 5, 10, and 15 min. Cells were rinsed with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, and 1.47 mM KH₂PO₄, pH 7.2), and cell lysates were collected following the manufacturer’s instructions (Kinexus, Vancouver, British Columbia, Canada). Pooled KSHV-infected and mock-infected samples were examined for phosphorylated proteins by using Kinex KAM-1.2FN 800 Ab antibody microarrays (Kinexus). Briefly, 50 µg of cell lysate from each sample was covalently labeled with a proprietary fluorescent dye (Kinexus). Free dye molecules were then removed by gel filtration at the completion of labeling reactions. After blocking of nonspecific binding sites, an incubation chamber was mounted onto the microarray to permit the loading of the KSHV-infected and mock-infected samples side by side on the same chip and to prevent mixing of the samples. Following sample incubation, unbound proteins were washed away. Each array produced a pair of 16-bit images, which were captured with a Perkin-Elmer ScanArray Reader laser array scanner (Waltham, MA). Signal quantification was performed with LimaGene 8.0 from BioDiscovery (El Segundo, CA), using the predetermined settings for spot segmentation and background correction. The array signal in each sample was corrected to the array background and log₂ transformed, and a Z score was calculated using the following formula: Z = (X - µ) / σ, where Z is the Z score, X is the value of the sample, µ is the population mean, and σ is the standard deviation. Thus, the Z score (or standard score) is a statistical measurement of a sample value’s relationship to the mean. It indicates how many standard deviations a sample value is from the mean. The Z ratio was further calculated by taking the difference between the average observed protein Z scores for the mock-infected and KSHV-infected groups and dividing it by the standard deviation of all the differences for that particular comparison. Kinases or signaling molecules were considered to be hits if the calculated Z ratio was ≥1 or ≤−1 and the variation between adjacent duplicate spots of the same antibody (% error range) was less than 30%. All the hits, together with previously published kinases and signaling molecules activated during primary KSHV infection, were analyzed for the enrichment of networks/pathways by Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA).

**Immunofluorescence assay (IFA).** For the detection of CREB1 activation following primary KSHV infection, HUVEC infected with KSHV for specified times were fixed in 4% paraformaldehyde for 10 min. Following three washes with PBS, the cells were incubated with a rabbit anti-phospho-CREB1 monoclonal antibody (Cell Signaling Technology) at 1:800 dilution for 60 min. The cells were then washed three times with PBS, followed by incubation with an Alexa Fluor 568-conjugated goat anti-rabbit IgG secondary antibody for 60 min. The cells were again washed with PBS three times and then stained with 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were visualized with a Nikon Eclipse Ti fluorescence microscope (Nikon Instruments, Inc., Melville, NY).

The KSHV LANA protein was detected as previously described (48), with minor modifications. At 48 h postinfection, KSHV-infected cells were fixed in methanol for 10 min. Following three washes with PBS, the cells were incubated with a rat anti-LANA monoclonal antibody (Abcam) at 1:500 dilution for 60 min. The cells were then washed three times with PBS, followed by incubation with an Alexa Fluor 568-conjugated goat anti-rat IgG secondary antibody for 60 min. The cells were again washed with PBS three times and then stained with DAPI.

The method for examining virus entry and trafficking was described previously (12). Briefly, following shRNA knockdown of CREB1 or MSKs or treatment with an MSK inhibitor, the cells were inoculated with virus and incubated for 6 h in the presence of the inhibitors, fixed in 2% paraformaldehyde, and processed for immunostaining of KSHV particles by use of an anti-Orf65 antibody. The numbers of virus particles docked at the perinuclear regions were calculated.

**Western blot analysis.** Cells infected with KSHV were collected for analysis at the specified times. Total protein preparations were separated in sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and detected with antibodies. Specific signals were revealed with chemiluminescence substrates and recorded using a UVP MultiSpectral imaging system (UVP LLC, Upland, CA).

**RT-qPCR.** The expression levels of viral genes were analyzed by reverse transcription–quantitative real-time PCR (RT-qPCR) using previously described procedures (17). Briefly, total RNAs from KSHV-infected HUVEC were prepared with Tri reagent as recommended by the manufacturer (Sigma). The RNA was treated with RNase-free DNase (Thermo Fisher Scientific, Inc., Waltham, WA) and reverse transcribed to obtain the first-strand cDNA by use of the Maxima reverse transcriptase system (Thermo Fisher Scientific, Inc.). For each sample, a control without reverse transcriptase was conducted in parallel. qPCR was then performed with the cDNA, using the gene-specific PCR primers described in **Table 1**. α-Tubulin was used as the internal control. Each sample was assayed in three repeats.

**shRNA knockdown.** shRNA plasmids were constructed by inserting annealed oligonucleotides containing the shRNA sequences specific for the target genes into the EcoRI and AgeI sites downstream of the U6 promoter in the pLKO.1 vector (Sigma-Aldrich). Recombinant lentivir-
ruses carrying shRNAs were produced by cotransfecting 293T cells with a mixture of plasmid DNAs consisting of pMD-G (vesicular stomatitis virus glycoprotein [VSV-G] envelope), pCMV-H9274R8.91 (Gag/Pol/Rev), and the pLKO/1-shRNA vectors (Aldevron, Fargo, ND), using the Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Virus-containing culture supernatants were collected 2 days after transfection and concentrated.

Knockdown with shRNAs was performed as previously described (49). Briefly, HUVEC were infected with shRNA-carrying lentiviruses in the presence of 10 μg/ml Polybrene (Sigma-Aldrich). Stable cell lines were selected by culturing the cells with 1.0 μg/ml puromycin (Calbiochem, La Jolla, CA) for 5 days. Western blotting or RT-qPCR was used to determine the effect of shRNA knockdown. The CREB1 shRNA knockdown sequences were GCCTGCAAACATTAACCATGA for shRNA1 and ACGG TGCCAACTCCAATTTAC for shRNA2; the MSK shRNA knockdown sequences were as follows: AGCAACCTTCCACGCCTTTAA (MSK1) and GCCACCTTCATGGCATTCAAC (MSK2) for shMixed1 and ACCT ATGACTTGGTGGAAAT (MSK1) and GCAGGGTGTTACCGAGGAAAGC (MSK2) for shMixed2.

RESULTS

Kinex antibody array assay identifies signaling molecules activated at early time points during primary KSHV infection. We conducted an antibody-based screen using a Kinex antibody microarray to identify kinases and signaling molecules that had significant changes in phosphorylation levels at early time points during primary KSHV infection. KSHV infection of HUVEC is productive, resulting in an active viral lytic transcriptional program and the production of infectious virions (16, 17). Serum-starved HUVEC were mock infected or infected with KSHV for 5, 10, and 15 min. We selected three time points in order to capture the phosphorylation and dephosphorylation events that occurred immediately following KSHV infection. Cell lysates collected at

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<th>Table 1 Sequences of primers used for RT-qPCR</th>
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<tr>
<td>Gene</td>
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<tr>
<td>CREB1</td>
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<td>α-Tubulin</td>
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<td>Orf50 (RTA)</td>
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<td>Orf-K8 (K-bZIP)</td>
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<td>Orf73 (LANA)</td>
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<th>Table 2 Kinex antibody microarray analysis results</th>
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<td>Target protein</td>
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<td>CREB1</td>
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<td>ZAP70/Syk</td>
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FIG 1  Signaling networks activated during primary KSHV infection as analyzed by Kinex antibody array and IPA. (A to C) Signaling networks activated during primary KSHV infection. Serum-starved HUVEC were mock infected or infected with KSHV for 5, 10, and 15 min. Cell lysates were collected, pooled, and analyzed with a Kinex antibody microarray. The antibody array data shown in Table 2, as well as kinases or signaling molecules that were previously reported to be activated during primary KSHV infection, were used as inputs and analyzed by IPA. The top enriched networks and their associated pathways were the integrin/MAPK network (A), the insulin/EGFR network (B), and the JAK/STAT network (C). Kinases or signaling molecules whose phosphorylation levels were increased by KSHV infection are shown in red, while those with a decreased phosphorylation status are shown in green, with the color intensities correlating with the levels of changes. The kinases or signaling molecules that were previously reported to be activated by KSHV infection are shown in gray, while those that were not known to be regulated by KSHV during primary infection are shown in white. ECM, extracellular matrix.
different time points were pooled and examined with the antibody array. We considered a kinase or signaling molecule to be a hit if its Z ratio, which reflected the difference in phosphorylation levels between KSHV-infected and mock-infected cells, was \( \geq 1 \) or \( \leq -1 \) and the variation between adjacent duplicate spots of the same protein was less than 30%. Based on these criteria, we found that a total of 23 unique phospho-sites of 18 proteins were increased, while 5 unique phospho-sites of 5 proteins were decreased, following KSHV infection (Table 2). These hits included PKB/AKT, integrin \( \beta_1 \), and c-Jun, whose roles in primary KSHV infection were reported previously (5, 9, 15). The roles of the other hits in primary KSHV infection had not been reported before, though PKC\( \delta \) was shown to mediate KSHV reactivation (23). These kinases and signaling molecules might potentially mediate different events during primary KSHV infection.

To place the identified hits in their corresponding signaling pathways, we performed IPA. Since the Kinex analysis captured only snapshots of the signaling cascades, we included kinases and signaling molecules that were reported to be activated during primary KSHV infection, such as FAK, SRC, ERK, and p38, in the pool of molecules used for network/pathway analysis. We found that integrin-MAPK, insulin-epidermal growth factor receptor (insulin-EGFR), and Janus kinase-signal transducer and activator of transcription (JAK/STAT) networks/pathways were enriched upon KSHV infection (Fig. 1A to C). The activation of integrin-MAPK pathways during primary KSHV infection has been stud-
ied extensively, thus confirming the overall validity of the approach. Nevertheless, a number of MAPKs, such as ERK, p38, and JNK, were not picked up, although their downstream effectors, such as c-Jun, CREB, and HSP27, were in the hit list (Fig. 1A), thus also revealing the limitation of this approach.

The insulin-EGFR pathways were previously shown to be involved in KSHV latency (50). However, their roles in primary KSHV infection had not yet been explored. The JAK/STAT pathways are complex and are involved in interferon or cytokine signaling. Because JAK1 and JAK2 were activated, while STAT5A was

FIG 3 CREB1 is required for the production of infectious virions but not for virus entry, trafficking, and infectivity during primary KSHV infection. (A) shRNA knockdown of CREB1. HUVEC were infected with lentviruses expressing shRNAs targeting CREB1 (shRNA1 or shRNA2) or expressing control shRNA sequences (Ctrl). Cells were selected with puromycin (1 μg/ml) and analyzed by Western blotting. (B and C) shRNA knockdown of CREB1 inhibits the production of infectious virions. HUVEC with knockdown of CREB1 were infected with KSHV. At 4 h postinfection, cells were extensively washed and replaced with new medium. At day 4 postinfection, the supernatants were collected and used to titrate infectious virions by infecting fresh HUVEC. (B) Representative images showing GFP-positive cells. (C) Similar images were used for quantification. (D) Representative images showing docking of viral particles on the nucleus of a HUVEC with knockdown of CREB1 or that of a control cell. HUVEC with knockdown of CREB1 or control cells were infected with KSHV for 6 h, fixed, and stained for the nucleus (blue) and for KSHV particles (with an antibody to Orf65) (red). (E) shRNA knockdown of CREB1 has no effect on virus entry and trafficking. The total number of KSHV particles successfully docked at each nucleus was quantified and analyzed based on images similar to those in panel D. The results are shown as box-and-whisker plots, with the open boxes representing the 75th and 25th percentiles, the top and bottom short lines representing the 90th and 10th percentiles, respectively, the middle thick lines in the boxes representing the medians, and the open circles outside the 90th and 10th percentiles representing the outliers. N, total number of cells analyzed per sample. (F) shRNA knockdown of CREB1 has no effect on the expression level and pattern of the KSHV latent protein LANA or on infectivity. HUVEC with knockdown of CREB1 were infected with KSHV, fixed at day 2 postinfection, and stained for LANA. The expression level and pattern of LANA are shown in the top panels, while the number of LANA-positive cells was quantified and is shown in the bottom panel. *, **, and ***, P values of <0.05, <0.01, and <0.001, respectively; NS, not significant.
controversial. While the total CREB1 level did not show a significant change at 15 and 30 min but had started to decrease at 60 min postinfection. The level of phospho-S133 CREB1 was increased levels of phospho-Ser133 and total CREB1 in HUVEC following stages of primary KSHV infection. The activation of CREB1 following KSHV infection. Furthermore, UV-inactivated virions activated CREB1 as efficiently as the wild-type (WT) virions did (Fig. 2B and C). These results suggested that KSHV-induced CREB1 phosphorylation was independent of viral gene expression but was mediated through the interactions between KSHV envelope glycoproteins and host cell receptors during the attachment and entry stages of infection.

shRNA knockdown of CREB1 inhibits the KSHV lytic replication program but has no effect on virus entry and trafficking, expression of the latent gene LANA, and KSHV infectivity. To explore the role of CREB1 in primary KSHV infection, we performed shRNA knockdown of CREB1. The Western blot results showed that CREB1 expression was reduced by over 70% in cells transduced with CREB1-specific shRNA-carrying lentiviruses compared to those transduced with the scrambled control-carrying lentivirus (Fig. 3A). CREB1 knockdown cells were infected with KSHV and extensively washed with culture medium at 4 h postinfection to remove any residual input viruses. At day 4 postinfection, the supernatants were collected and titrated. Knockdown of CREB1 reduced virus titers by approximately 70% for both shRNAs (Fig. 3B and C). These results indicated that CREB1 plays an important role in KSHV lytic replication and virion production during primary infection.

KSHV productive infection is regulated at different steps during primary infection, including virus entry, trafficking, and viral gene expression. To identify the step(s) mediated by CREB1, we first examined KSHV entry and trafficking to the cell nucleus. HUVEC with knockdown of CREB1 were infected with KSHV for 6 h. Cells were extensively washed, fixed, and stained for the Orf65 protein to visualize the viral capsids that had successfully trafficked to the perinuclear region. By counting viral capsids docking at the nucleus, we found that similar numbers of virus particles had successfully reached the cell nucleus in both control and CREB1 knockdown cells (Fig. 3D and E). These results indicated that CREB1 was not involved in virus entry and intracellular trafficking.

We further determined if knockdown of CREB1 might affect KSHV infectivity. Because LANA is the major KSHV latent protein that binds to the viral genome to maintain its persistence, detection of the LANA protein, which manifests as a punctate staining pattern, would indicate successful viral infection. HUVEC transduced with lentiviruses carrying CREB1-specific or control shRNAs were infected with KSHV and stained for the LANA protein at day 2 postinfection. Knockdown of CREB1 had no effect on the LANA staining pattern and intensity, the number of punctate dots per cell, and the percentage of LANA-positive cells (Fig. 3F), indicating that CREB1 did not affect LANA protein expression and KSHV infectivity. Taken together, the results show that CREB1 is an essential factor for viral lytic replication and virion production but that it neither regulates viral entry and trafficking nor affects KSHV infectivity during primary infection.

Knockdown of CREB1 inhibits the expression of KSHV lytic but not latent genes. Based on their distinct expression kinetics, KSHV genes are classified into latent and lytic genes. Latent genes, which mainly consist of LANA, vFLIP, vCyclin, and a cluster of KSHV genes are classified into latent and lytic genes. Latent genes, which mainly consist of LANA, vFLIP, vCyclin, and a cluster of microRNAs, promote cell growth and survival and viral latency (51). Lytic genes, consisting of immediate early (IE), early (E), and late (L) genes, are expressed in a coordinated fashion during KSHV lytic replication. The expression of the IE RTA gene is necessary and sufficient for activating KSHV into full lytic replication (51). The early gene Orf-K8 encodes another important regulator of lytic replication, K-bZIP. The late genes, such as Orf65 and
FIG 5 Chemical activation of CREB1 with forskolin (FSK) promotes KSHV lytic replication but has no effect on virus entry, trafficking, and infectivity during primary infection. (A) FSK activates the PKA/CREB1 pathway. HUVEC were treated with FSK for 1 h and then examined for phospho-CREB1 (S133), total CREB1, phospho-PKA (S338), and total PKA. (B) Activation of CREB1 with FSK increases the production of infectious virions. HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK. At 4 h postinfection, cells were extensively washed and replaced with new medium containing FSK. At 48 h postinfection, the supernatants were collected and subjected to titration of infectious virions as described in the legend to Fig. 3B and C. NT, not treated. (C) Activation of CREB1 with FSK has no effect on virus entry and trafficking. HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK for 6 h. The number of KSHV particles successfully docked at
Orf39 (glycoprotein M), are mostly composed of genes encoding viral structural proteins and those required for viral lytic replication, and their expression often depends on viral genome replication. During primary KSHV infection of HUVEC, both latent and lytic genes are actively transcribed, with most of them peaking at 48 to 72 h postinfection (17). The results of immunostaining showed that shRNA knockdown of CREB1 did not affect LANA protein expression (Fig. 3F). To test the role of CREB1 in viral lytic gene expression during primary infection, we monitored the kinetics of several representative KSHV lytic genes by RT-qPCR. Knockdown of CREB1 inhibited over 90% of the expression of the RTA, K-bZIP, and Orf65 lytic genes (Fig. 4A to C) but had no significant effect on the expression of the latent gene LANA (Fig. 4D), which was in agreement with the immunostaining results (Fig. 3F). Western blot analysis further confirmed that knockdown of CREB1 decreased over 90% of the expression of the RTA, K-bZIP, and Orf65 viral lytic proteins but had no effect on the expression of the viral latent protein LANA (Fig. 4E). Taken together, these results suggest an essential role of CREB1 in the viral lytic transcriptional program.

Activation of CREB1 is sufficient to promote KSHV lytic replication during primary infection. Next, we determined if artificial activation of CREB1 was sufficient to promote viral lytic replication during primary infection. Since there is no chemical agonist that can directly activate CREB1, we chose an indirect approach by using the PKA agonist forskolin (FSK). FSK raises the intracellular cAMP level by activating adenylyl cyclase, resulting in the activation of the PKA pathway, the classic upstream pathway of CREB1 (24). Following pretreatment of cells with 10 μM FSK, we infected HUVEC with KSHV in the presence of the agonist. FSK increased the phosphorylation level of PKA at S338 and activated CREB1 (Fig. 5A). Consistent with these results, FSK enhanced the production of infectious virions 2.7-fold (Fig. 5B), indicating that activation of CREB1 alone was sufficient to significantly promote viral lytic replication during primary infection. Similar to knockdown of CREB1, FSK treatment did not affect virus entry and trafficking, had no effect on the expression level and staining pattern of LANA, and conferred a minimal effect on KSHV infectivity (Fig. 5C and D). Consistent with its enhanced effect on the production of infectious virions, FSK significantly enhanced the mRNA expression of the KSHV RTA, K-bZIP, and Orf65 lytic genes, increasing their expression levels 18-, 450-, and 288-fold, respectively, at 40 h postinfection (Fig. 5E to G). Interestingly, FSK also increased the expression of LANA, but only 4.6-fold (Fig. 5H). In agreement with the mRNA results, FSK increased the expression levels of the RTA, K-bZIP, and Orf65 lytic proteins (Fig. 5I). The LANA protein level was also increased by FSK, albeit to a lower level. Taken together, these results show that chemical activation of CREB1 was sufficient to increase viral lytic replication during primary KSHV infection, predominantly by enhancing the expression of viral lytic genes.

Screening of CREB1’s direct effectors identifies MSK1 and -2 as upstream CREB1 regulators during primary KSHV infection. Having defined the role of CREB1 in primary KSHV infection, we next attempted to identify the cellular pathway(s) that directly mediated CREB1 activation. The classic CREB1 activation pathway is the cAMP/PKA pathway (24). As a putative protein kinase for CREB1 activation, activation of PKA as a result of an increased second messenger (cAMP) level leads to CREB1 phosphorylation at Ser133 (39). We first measured the change in cAMP level during primary KSHV infection. Using a specific anti-cAMP antibody in an enzyme-linked immunosorbent assay (ELISA), we did not detect any significant change in the cAMP level within the first 1 h of primary KSHV infection (Fig. 6A), during which time CREB1 was activated (Fig. 6B). As expected, FSK drastically raised the intracellular cAMP level (Fig. 6A). Consistent with these results, we did not detect any change of PKA phosphorylation when CREB1 was activated at 15 and 30 min post-KSHV infection (Fig. 6B). Using a specific antibody against the PKA catalytic subunit (PKAc), we did not detect any cytoplasmic-to-nuclear translocation of PKA at these time points (Fig. 6C). Taken together, these results indicated that the cAMP/PKA pathway was not involved in KSHV-induced CREB1 activation during primary infection, although it could actively promote the KSHV lytic transcriptional program if artificially activated (Fig. 5). This finding was contradictory to that of a previous study showing that KSHV activation of CREB1 was mediated by the cAMP/PKA pathway in DMVEC (35), indicating that KSHV activation occurs via distinct signaling pathways in different cell types.

Previous studies revealed that besides PKA, several other protein kinases can directly phosphorylate CREB1. These kinases include AKT, RSKs, MSKs, and MK2. We first checked the activation of these kinases during primary KSHV infection. The Western blot results showed that AKT, MSK1/2, and MK2, but not RSKs, were phosphorylated at the early time points of KSHV infection (Fig. 7A). Similar to the case for CREB1, the increased phosphorylation levels of AKT, MSK1/2, and MK2 peaked at around 15 to 30 min postinfection, suggesting their possible involvement in CREB1 activation. We then used specific inhibitors to investigate the involvement of these kinases in CREB1 activation. LY294002 is an inhibitor of the AKT upstream kinase PI3K, SB747651A is an inhibitor of MSK1/2, and PF3644022 is an inhibitor of MK2. HUVEC were preincubated with the individual inhibitors at 37°C for 1 h, at final concentrations of 12.5 μM, 10 μM, and 10 μM for LY294002, SB747651A, and PF3644022, respectively, and then infected with KSHV in the presence of the inhibitors. Western blot analysis of the infected cells showed that the inhibitor of MSKs, but not that of PI3K or MK2, prevented CREB1 activation (Fig. 7B to D). The inhibitor of MSKs reduced the phosphorylation levels of CREB1 by 77% and 73% at 15 and 30 min postinfection, respectively (Fig. 7B). In contrast, inhibitors of PI3K and MK2 not only did not decrease but in fact increased the nucleus as was analyzed as described in the legend to Fig. 3D and E. (D) Activation of CREB1 with FSK has no effect on the expression level and pattern of the KSHV latent protein LANA or on infectivity. HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK and analyzed for the expression level and pattern of LANA and the number of LANA-positive cells at 48 h postinfection. (E to H) Activation of CREB1 with FSK significantly enhances the mRNA expression of the KSHV RTA (E), K-bZIP (F), and Orf65 (G) lytic genes but has a lesser effect on the latent gene LANA (H). HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK and analyzed for the mRNA expression of viral genes by RT-qPCR at 15, 24, and 40 h postinfection. (I) Activation of CREB1 with FSK increases the expression of the KSHV RTA, K-bZIP, and Orf65 lytic proteins but increases that of the LANA protein to a lesser extent. HUVEC pretreated with FSK for 1 h were infected with KSHV in the presence of FSK and analyzed for the expression of viral proteins by Western blotting at 48 h postinfection. *", **", and "***", P values of <0.05, <0.01, and <0.001, respectively; NS, not significant.
CREB1 activation, even before KSHV infection (0 h). We successfully detected reduced phosphorylation levels of AKT and the MK2 downstream target Hsp27, confirming that the two inhibitors were functional (Fig. 7C and D). Together, these results showed that KSHV-induced activation of CREB1 was mediated by MSKs but not by AKT or MK2.

MSK1 and -2 regulate KSHV productive lytic replication during primary infection, but not virus entry, intracellular trafficking, and KSHV infectivity. To determine the role of MSK1/2 in primary KSHV infection, we infected HUVEC in the presence of the MSK inhibitor SB747651A (10 μM). Inhibition of MSKs decreased the production of infectious virions by approximately 62% (Fig. 8A), indicating an essential role of MSKs in productive KSHV replication during primary infection. On the other hand, inhibition of MSKs did not affect the number of viral particles reaching the perinuclear region, indicating that MSKs did not regulate KSHV entry and intracellular trafficking (Fig. 8B). Furthermore, MSKs did not affect the expression and staining pattern of LANA or KSHV infectivity (Fig. 8C). The results of RT-qPCR analysis showed that inhibition of MSKs reduced the expression of RTA, K-bZIP, and Orf65 transcripts by 56 to 61%, 43 to 50%, and 32 to 35%, respectively, but had no effect on the expression of LANA transcripts (Fig. 8D). The Western blot results confirmed those of RT-qPCR, showing that MSK shRNAs reduced the expression of the RTA, K-bZIP, and Orf65 proteins but had no effect on the LANA protein (Fig. 8F). Taken together, the results show that MSKs play an important role in regulating viral lytic replication during primary KSHV infection and that such regulation might occur at least partially through phosphorylating and activating the downstream transcription factor CREB1.

**DISCUSSION**

Deregulation of cell signaling pathways is a common theme in viral infections. Viruses often hijack cellular pathways to achieve successful infection and replication. For example, the MSK-CREB1 pathway has been reported to mediate ERK-induced HCMV reactivation in dendritic cells (44). CREB1 activation promotes HCMV gene expression by binding to the viral major immediate early promoter. Similarly, HSV-1 depends on CREB1 to regulate the expression of an 8.3-kb primary latency-associated transcript during acute and latent infections of peripheral sensory neurons.
In this study, we applied an unbiased antibody array approach to identify new kinases or signaling molecules that might play important roles during primary KSHV infection. The Kinex antibody array has a relatively broad coverage of human kinases and signaling molecules. We were interested in kinases and signaling molecules that might be deregulated early during primary KSHV infection, as they might mediate virus attachment, entry, membrane fusion, intracellular trafficking, and viral gene expression. Using stringent criteria, we identified 28 unique phospho-sites from 23 cellular proteins whose phosphorylation levels were significantly altered at the early phases of KSHV infection of HUVEC (Table 2). A number of these hits, including AKT, integrin β1, and c-Jun, were previously reported (5, 9, 15). It would be interesting to confirm the alterations of the newly identified hits and to explore their potential roles in primary KSHV infection.

By performing IPA with the Kinex antibody array results integrated with those reported previously, we identified three enriched networks. All three networks are initiated as a result of ligand-receptor interactions. In this case, they might be due to KSHV binding to its receptors or cytokines/growth factors induced at the early stage of KSHV infection. The first is the integrin/MAPK network (Fig. 1A), parts of which have been reported in several studies (5, 9, 10, 15). Nevertheless, the upstream activators, the downstream mediators, and the interactions among the signaling pathways in this network in the context of primary KSHV infection remain unclear. The current paradigm is that KSHV binding to cellular integrin receptors activates downstream FAK (5), which can lead to the induction of multiple MAPK pathways and the PI3K/AKT pathway (9, 10, 15, 52). Activation of the ERK, JNK, and p38 MAPK pathways promotes KSHV entry and trafficking as well as the viral lytic replication program (9, 52). The AKT pathway mediates activation of the NF-κB pathway, which regulates KSHV latent gene expression (15). While KSHV infection triggers the immediate release of proinflammatory and proangiogenic cytokines, such as IL-6, angiopoietin-2, and IL-8 (10, 53), a number of them, including IL-6, angiopoietin-2, MMP1, MMP2, and MMP9, can also be induced further by the MAPK pathways (10, 54, 55). Importantly, some of these cytokines in turn can activate the ERK, AKT, and NF-κB pathways, thus allowing the establishment of a positive-feedback loop that can further enhance KSHV infection and replication and amplify a proinflammatory and proangiogenic environment during primary KSHV infection.

Another enriched network involved the insulin receptor and EGFR pathways (Fig. 1B). The insulin-like growth factor and insulin receptor signaling pathways have been implicated in latent KSHV infection (30). However, the roles of the insulin receptor and EGFR in primary KSHV infection are still unknown. EGFR is a receptor tyrosine kinase that regulates a number of key cellular processes, including cell proliferation, survival, differentiation, tissue homeostasis, and tumorigenesis. EGFR and ephrin receptor A2 (EphA2A) have been identified as hepatitis C virus (HCV) entry receptors (56). KSHV also uses EphA2A as an entry receptor for infection (57). Binding of glycoproteins gH and gL to EphA2 trig- ers EphA2 phosphorylation and endocytosis and KSHV entry (57). It would be interesting to explore the role of EGFR in primary KSHV infection.

The JAK/STAT network was enriched in the IPA results (Fig. 1C). In particular, the phosphorylation levels of JAK1 and JAK2 increased >4-fold. A number of proinflammatory cyto-
Chemical inhibition of the MSK pathway blocks the KSHV lytic replication program but has no effect on virus entry, trafficking, and infectivity during primary infection. (A) Inhibition of the MSK pathway blocks the production of infectious virions. HUVEC were pretreated with the MSK inhibitor SB747651A for 1 h and then infected with KSHV in the presence of the inhibitor. At 4 h postinfection, cells were extensively washed and replaced with new medium containing SB747651A for 1 h.

(B) SB747651A inhibits viral gene expression. HUVEC were infected with KSHV for 1 h in the presence of SB747651A. At 21 hpi, the nuclear DNA was isolated and subjected to quantitative PCR analysis. Gene expression is presented as fold change with NT as the calibrator. Statistical significance was determined by Student's t test (**p < 0.01, ***p < 0.001).
kines, such as IL-6, are known to activate JAK1 and JAK2 (58). However, IL-6 primarily activates the STAT3 pathway (59). We did not observe any significant change in STAT3 phosphorylation during primary KSHV infection, although STAT3 is implicated in KSHV latent infection (60, 61) and can be activated by KSHV vIL6, RTA, vGPCR (ORF74), and kaposin B, as well as through complement activation (61–65). In contrast to that of JAK1/2, the phosphorylation level of STAT5A was significantly reduced. STAT5A is primarily activated by growth factors, such as prolactin (66). Further studies are required to understand how these pathways are regulated during primary KSHV infection.

Several kinases, such as ERK, AKT, and FAK, were reported to be activated during primary KSHV infection but were not identified as hits in the screen. This could be attributed to the limitations of the antibody array approach, which can generate both false-positive and false-negative candidates. Possible limitations of the approach include the following. (i) Antibodies to some targets/phosphorylation sites are absent on the array. (ii) Some antibodies are of poor quality or are not suitable for the antibody array. (iii) Some antibodies have limited sensitivities. (iv) Some antibodies have high variations between adjacent duplicate spots, which could lead to the discard of some hits. For example, the p38 phosphorylation sites T180 and Y182 had the highest signal, with a Z ratio of 6.08, but were discarded due to the high (79.16%) error range (data not shown). (v) Kinase activation is a fast event with rapid kinetics. The collected samples reflect only snapshots of the cells and might not catch all events. Because of the reasons stated above, it is imperative to validate any selected hits following antibody array screening. Despite these limitations, the antibody array assay remains an easy and convenient approach that can generate some potential hits. Combining this method with IPA or similar bioinformatic analyses and other approaches, such as gene expression profiling, should facilitate the mapping of the overall networks and pathways that are activated in the cells.

We have identified MSK1/2-CREB1 as a new essential pathway for KSHV lytic replication during primary infection. Chemical inhibition of MSK1/2 or shRNA knockdown of MSK1/2 or CREB1 substantially impaired the expression of viral lytic genes and the production of infectious virions but had no effect on virus entry, trafficking, latent gene expression, and infectivity. One possible mechanism of CREB1 promotion of KSHV lytic replication is through its binding to the promoters of viral lytic genes to induce their expression. In fact, a CRE site is present from bp −265 to −269 upstream of the transcriptional start site in the RTA gene promoter. As RTA is the major driver of the viral lytic cascade, induction of RTA is sufficient to enhance KSHV lytic replication. The promoters of several other KSHV lytic genes, including the Orf59, K-bZIP, and Orf65 genes, also contain putative CRE sites, which may mediate CREB1’s enhancement of KSHV lytic replication. On the other hand, we did not find any potential CRE site in the LANA promoters, which might explain the differential responses between LANA and viral lytic genes. It is worth mentioning that CREB1 regulation of gene expression might be gene or promoter specific and could be influenced by other transcriptional factors. For example, it has been shown that CREB1 activation is enhanced by RTA for promoters containing an RTA-binding site(s); however, it is inhibited by RTA for promoters without an RTA-binding site, in part through competition or sequestration of other transcription coactivators, such as CBP (67). Thus, CREB1 and RTA might synergize to drive the expression of KSHV lytic genes while antagonizing each other to regulate the expression of latent genes during primary infection of HUVEC. However, it has been reported that RTA can activate the LANA promoter through an RBP-Jκ binding site (68). Thus, the lack of responsiveness of LANA expression to CREB1 activation is unlikely to be mediated by RTA.

Another possible mechanism of CREB1 regulation of viral lytic replication is through the induction of proinflammatory cytokines, several of which promote KSHV lytic replication (38). However, it has been reported that CREB1 induces miR-132 to downregulate p300, the proinflammatory cytokines IL-6, IL-1β, and beta interferon, and ISG15, relieving their repression of the expression of KSHV genes and the viral genome copy number in LEC (37). It is interesting that two of the CREB1-downregulated proinflammatory cytokines, IL-6 and IL-1β, can activate the MAPK pathways and promote KSHV lytic replication (38). Another study has also shown that KSHV infection of DMVEC activates CREB1 to induce the proinflammatory factor COX2, which is required for viral latent gene expression (35). Interestingly, during primary KSHV infection of DMVEC, CREB1 activation occurs through the cAMP/PKA pathway but not the MSK pathway (35). It is also worth mentioning that primary KSHV infections in LEC and DMVEC are nonproductive despite the activation of CREB1 (19, 36).

Regardless of the mechanism of CREB1 promotion of KSHV lytic replication, the outcome of CREB1 activation in regard to KSHV lytic replication during primary infection differs depending on the cell type, which might be due to usage of different cellular receptors and entry pathways resulting in the differential activation of signaling pathways. In particular, KSHV induces a high level of activation of the NF-κB pathway in DMVEC (15), which is known to promote KSHV latency by repressing viral lytic replication (22). In contrast, KSHV induces hyperactivation of the ERK, JNK, and p38 MAPK pathways in HUVEC, promoting viral...
FIG 9 MSK1 and -2 are required for CREB1 activation and KSHV lytic replication but not for virus entry, trafficking, and infectivity during primary infection. (A) shRNA knockdown of MSK1/2 prevents CREB1 activation. HUVEC were infected with lentiviruses expressing 2 sets of combined shRNAs targeting both MSK1 and MSK2 (shMixed1 and shMixed2) or the control shRNA (Ctrl). Stable cell populations selected with puromycin (1 μg/ml) were infected with KSHV and analyzed by Western blotting for total MSK1, total MSK2, phospho-CREB1 (S133), and total CREB1 at the indicated times. (B) shRNA knockdown of MSK1/2 inhibits the production of infectious virions. HUVEC with knockdown of MSK1/2 were infected with KSHV. At 4 h postinfection, cells were extensively washed and replaced with new medium. At day 4 postinfection, the supernatants were collected and used to titrate infectious virions by infecting fresh HUVEC as described in the legend to Fig. 3Band C. (C) shRNA knockdown of MSK1/2 has no effect on virus entry and trafficking. HUVEC with knockdown of MSK1/2 were infected with KSHV for 6 h. The number of KSHV particles successfully docked at the nucleus was analyzed as described in the legend to Fig. 3D and E. (D) shRNA knockdown of MSK1/2 has no effect on the expression level and pattern of the KSHV latent protein LANA or on infectivity. HUVEC with knockdown of MSK1/2 were infected with KSHV and analyzed for the expression level and pattern of LANA and the number of LANA-positive cells at 48 h postinfection. (E) shRNA knockdown of MSK1/2 significantly blocks the mRNA expression of the KSHV RTA, K-bZIP, and Orf65 lytic genes but has a minimal effect on the latent gene LANA. HUVEC with knockdown of MSK1/2 were infected with KSHV and analyzed for the mRNA expression of viral genes by RT-qPCR at 48 h postinfection. (F) shRNA knockdown of MSK1/2 blocks the expression of the KSHV RTA, K-bZIP, and Orf65 lytic proteins but has no effect on the latent protein LANA. HUVEC with knockdown of MSK1/2 were infected with KSHV and analyzed for the expression of viral proteins by Western blotting at 48 h postinfection.

* * *, **, and ***P values of <0.05, <0.01, and <0.001, respectively; NS, not significant.
lytic replication (9, 10). The interaction of the MAPK and NF-κB pathways might be one of the factors that determines the impact of MSK1/2-CREB1 activation on the KSHV replication program. In this case, the level of MSK1/2-CREB1 activation might have only a minimal impact on the KSHV replication program. Alternatively, the KSHV replication program could be determined entirely by the extent of MSK1/2-CREB1 activation. The MSK1/2-CREB1 proteins could be the critical mediators of the hyperactivated MAPK pathways, in addition to the AP-1 complexes, which control KSHV lytic replication in HUVEC. In this scenario, MSK1/2-CREB1 could also be activated by other pathways/signals. Indeed, besides MSK1/2, CREB1 can also be activated directly by cAMP/PKA, PI3K/AKT, MK2, and RSK pathways. As a result, intracellular and extracellular signals that can activate CREB1 are likely to promote KSHV lytic replication. As predicted, we have shown that chemical activation of CREB1 with FSK, an artificial activator of the cAMP/PKA pathway, can robustly enhance viral lytic replication (Fig. 5). Our works have clearly identified CREB1 as an essential factor for KSHV lytic replication during primary infection of HUVEC. Because phosphorylation is required for CREB1 activation, further demonstration of the role of CREB1 phosphorylation in KSHV lytic replication would require mutation of the specific CREB1 residue that mediates its phosphorylation.

While our results have demonstrated only that the MSK1/2-CREB1 pathway promotes KSHV lytic replication during primary infection, it is likely that it also enhances KSHV reactivation from latency. In fact, CREB1 has been reported to mediate reactivation of several other herpesviruses from latency (44, 69, 70). In LPS-induced HCMV reactivation, activated CREB1 induces the expression of the viral immediate early gene by binding directly to the promoter (44). MSKs can also phosphorylate histone H3 at serine, which might contribute to the expression of the viral immediate early gene. In HSV-1-induced Epstein-Barr virus (EBV) reactivation, the PKA/CREB1 pathway mediates the activation of Zta, a major transcriptional transactivator of EBV lytic replication (69). A previous study of KSHV demonstrated that activation of the cAMP/PKA pathway alone is sufficient to reactivate KSHV from latency (34). Because CREB1 is one of the primary transcription factors that mediate PKA signaling (39), deletion of CRE from the RTA promoter reduced PKA-induced RTA expression by 50% (34). Thus, the cAMP/PKA/CREB1 pathway is essential for KSHV reactivation. It is interesting that the cAMP/PKA pathway is not activated during primary KSHV infection of HUVEC. Instead, the MSK1/2-CREB pathway is activated and contributes to the viral lytic replication program. Because MSKs can activate multiple transcription factors, including CREB and AP1, it would be worthwhile to study the role of the MSK pathway in KSHV reactivation.

In KS lesions, most of the spindle-shaped tumor cells are latently infected by KSHV, indicating an essential role of viral latent infection in KSHV-induced malignant cell proliferation (71–73). However, a small subset of KS tumor cells also undergo spontaneous viral lytic replication, which might contribute to KS development (51). In patients, KSHV lytic activity and the viral load are strongly correlated with the progression of KS tumors (74–77). Our results suggest that the MSK1/2-CREB1 pathway might be a potential target for inhibiting KSHV lytic replication. Since CREB1 signaling can also induce proinflammatory cytokines, including IL-6 and tumor necrosis factor alpha (TNF-α) (25), targeting the MSK1/2-CREB1 pathway could have dual inhibitory effects on KSHV lytic replication and inflammation by preventing the establishment of a vicious amplification cycle, particularly during the early stage of tumor development.

Figure 10 illustrates the confirmed cellular signaling pathways that are hijacked by KSHV for the optimal expression of viral genes and productive viral lytic replication during primary KSHV infection.

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