Modulation of Kaposi’s Sarcoma-Associated Herpesvirus Infection and Replication by MEK/ERK, JNK, and p38 Multiple Mitogen-Activated Protein Kinase Pathways during Primary Infection

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Kaposi’s sarcoma-associated herpesvirus (KSHV) is etiologically associated with Kaposi’s sarcoma, a dominant AIDS-related tumor of endothelial cells, and several other lymphoproliferative malignancies. While activation of the phosphatidylinositol 3-kinase–protein kinase C–MEK–ERK pathway is essential for KSHV infection, we have recently shown that KSHV also activates JNK and p38 mitogen-activated protein kinase (MAPK) pathways during primary infection (J. Xie, H. Y. Pan, S. Yoo, and S.-J. Gao, J. Virol. 79:15027–15037, 2005). Here, we found that activation of both JNK and p38 pathways was also essential for KSHV infection. Inhibitors of all three MAPK pathways reduced KSHV infectivity in both human umbilical vein endothelial cells (HUVEC) and 293 cells. These inhibitory effects were dose dependent and occurred at the virus entry stage of infection. Consistently, inhibition of all three MAPK pathways with dominant-negative constructs reduced KSHV infectivity whereas activation of the ERK pathway but not the JNK and p38 pathways enhanced KSHV infectivity. Importantly, inhibition of all three MAPK pathways also reduced the yield of infectious virions during KSHV productive infection of HUVEC. While the reduction of infectious virions was in part due to the reduced infectivity, it was also the result of direct modulation of KSHV lytic replication by the MAPK pathways. Accordingly, KSHV upregulated the expression of RTA (Orf50), a master transactivator of KSHV lytic replication, and activated its promoter during primary infection. Furthermore, KSHV activation of RTA promoter during primary infection was modulated by all three MAPK pathways, predominantly through their downstream target AP-1. Together, these results indicate that, by modulating multiple MAPK pathways, KSHV manipulates the host cells to facilitate its entry into the cells and postentry productive lytic replication during primary infection.

Infection by viruses alters signaling pathways as a result of cellular response to the infection and virus modulation of its environments. Viruses have evolved to depend on these altered cellular pathways for their successful infection and replication in the host cells. For example, modulation of mitogen-activated protein kinase (MAPK) pathways is essential for infection and replication of human immunodeficiency virus, hepatitis B virus, Epstein-Barr virus, and vaccinia virus (11, 23, 31, 54), while modulation of the NF-κB pathway facilitates infection and replication of Epstein-Barr virus, herpes simplex virus type 1, and influenza virus (22, 39, 44).

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is a gammaherpesvirus associated with Kaposi’s sarcoma (KS), primary effusion lymphoma, and multicentric Castleman’s disease (14). The life cycle of KSHV has two phases. The latent phase, during which the virus is maintained as episomes and has restricted expression of latent genes, is essential for the development of KSHV-induced malignancies (41, 55). The lytic phase, during which the virus produces infectious virions for dissemination, modulates cellular signaling pathways through unrestricted expression of viral genes (14).

KSHV infects a variety of cell types, including B cells, epithelial cells, keratinocytes, and endothelial cells. Although KSHV establishes latency in the majority of cell types following primary infection (29), we have found that efficient infection of human umbilical vein endothelial cells (HUVEC) is productive at the early stage of infection, producing large number of infectious virions preceded by strong expression of almost all viral lytic genes (19, 53).

KSHV entry into the host cells relies on the interaction of its envelope glycoprotein B (gB) with cellular receptor integrin α3β1 (3). This specific ligand-receptor interaction activates focal adhesion kinase and the MEK-ERK1/2 MAPK pathway but not the JNK and p38 MAPK pathways (3, 38). The activation of MEK pathway is important for KSHV infection, since specific inhibitors of MEK pathway reduce KSHV infectivity and the expression of KSHV early transcripts without affecting virus binding (38, 42). Consistently, overexpression of Raf, a component of MEK pathway, enhances KSHV infectivity at the postattachment stage (1). Recently, it has been shown that binding of KSHV virions to the cells is sufficient to activate the RTA (Orf50) promoter (32). These studies indicate a role for the MEK MAPK pathway in KSHV infection.

We have recently shown that besides the MEK MAPK pathway, KSHV infection also activates JNK and p38 MAPK path-
ways at the early stage of infection (50). These observations indicate that besides the interaction between gB and integrin α3β1, there are other viral ligand(s) and cellular receptor(s) that are involved in KSHV entry into the cells. In this study, we have examined the role of all three MAPK pathways in KSHV infection and replication during primary infection. Besides MEK pathway, we found that activation of both JNK and p38 pathways is essential also for efficient KSHV infection. More importantly, we have observed that all three MAPK pathways modulate KSHV lytic replication and production of infectious virions during primary infection. These results illustrate a mechanism by which KSHV manipulates the host cells to facilitate its infection and replication during primary infection.

MATERIALS AND METHODS

Plasmids. The RTA full-length reporter construct R-914 containing the 914- and 36-bp RTA promoter sequences upstream and downstream of the transcriptional start site (−542 to +2), respectively, cloned in a luciferase reporter vector pGL-3 was obtained from Koichi Yamamishi (9). A set of deletion constructs of the RTA promoter, including R-836, R-587, R-348, R-259, and R-15, were generated using R-914 as a template and the 36-bp RTA promoter sequences downstream of the transcriptional start site and 836, 587, 584, 259, and 15 bp of the RTA promoter sequences upstream of the transcriptional starting site, respectively (see Fig. 9). A mutant promoter reporter, R-259mut, was generated using R-259 as a template by site-directed mutagenesis with a QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA) to ablate the AP-1 site. The correct mutations of the wild-type AP-1 site from −8TCGACTCA-81 to −8TCGAGCTC-81 were verified by DNA sequencing.

The following plasmids were described before: pCEP4-ERK1 and pCEP4-HA-ERK1/K7IR are expression vectors for ERK1 and its dominant-negative (DN) construct, respectively (17); pcDNA-wt-p38 is an expression vector for wild-type p38 (45), and pcDNA3-p38/AF is a DN construct of p38 (27); HA-JNKpCAGGGS is a JNK1 expression construct with JNK1 cloned into the pCAGGS vector (18), and HA-JNK[APF] is a DN construct of JNK1 (12); A-Fos is a DN construct of c-Fos (40), and pcDNA3Flag-c-Fos expresses an active form of c-Fos (37); RSV-c-Jun expresses an active form of c-Jun, and Dnc-Jun is a DN construct of c-Jun (kindly provided by S. L. Li at the University of Texas Health Science Center at San Antonio, San Antonio, TX).

Cell culture. Human embryonic kidney 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml gentamicin, and 2 mM l-glutamine. Primary HUVEC were purchased commercially and cultured in endothelial cell growth medium EB2M (Clonetics, Walkersville, MD).

Virus preparation and infection. A volume of concentrated virus was prepared from recombinant KSHV BAC36-infected 293 or BCBL-1 cells as previously described (19, 56). Briefly, supernatant from O-tetradecanoylphorbol 13-acetate (Sigma, St. Louis, MO)-induced cells was first centrifuged twice at 5,000 × g for 10 min to eliminate cell debris and then at 100,000 × g for 1 h with 20% sucrose as a cushion. The final pellet was dissolved in culture medium overnight. Fresh virus preparations with titers of about 2 × 10⁷ infectious particles/ml (GFU) were used in the experiments. HUVEC or 293 cells were infected with virus as previously described (19, 56). For all experiments, cells were incubated at 37°C in 5% CO₂ for 24 h prior to treatment with virus as described. For UV light treatment, virus preparations were exposed to a UV source for 5 min, which reduced virus infectivity from 70% to 80% to less than 1%. For inhibition of a MAPK pathway during primary infection, an inhibitor specific for a pathway was added 30 min prior to infection at the 0 h time point and at different time points after infection for the time course study: U0126 (10 μM), an inhibitor of MEK; SB203580 (50 μM), an inhibitor of p38; and JNK inhibitor II (50 μM), an inhibitor of JNK (all purchased from Calbiochem, Oakland, CA). Different concentrations of each inhibitor were used in the dose experiments. To determine the effects of inhibiting MAPK pathways on KSHV latency, BAC36-lentently infected 293 cells, HUVEC, and BCBL-1 cells were continuously treated with inhibitors of MAPK pathways for 5 days with daily changes of fresh medium and inhibitors. The percentages of cells expressing GFP and latent nuclear antigen (LANA) were then determined after the 5-day treatment.

Immunofluorescence assay. The expression of KSHV LANA encoded by Orf73 was detected as previously described (21) with minor modifications. KSHV-infected cells were fixed in 1% paraformaldehyde at room temperature for 10 min. Following three washes with phosphate-buffered saline (PBS) containing NaCl at 137 mM, KCl at 2.68 mM, Na₂HPO₄ at 8.1 mM, and KH₂PO₄ at 1.47 mM (pH 7.2), the cells were incubated with a rat anti-LANA monoclonal antibody (ABI, New York, NY) at a 1:100 dilution for 40 min at room temperature. The cells were then washed three times with PBS followed by incubation with a rhodamine-conjugated goat anti-rat immunoglobulin G secondary antibody for 30 min at room temperature. The cells were again washed with PBS three times and stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Cells expressing LANA with a speckle nuclear pattern were visualized with a Zeiss Epi fluorescence microscope (Carl Zeiss, Thornwood, NY).

Western blot analysis. Protein preparations from mock- and KSHV-infected cells were separated in sodium dodecyl sulfate-polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes as previously described (20). The membranes were incubated first with antibodies specific for phosphorylated forms of JNK, ERK1/2, and p38 (Santa Cruz, Santa Cruz, CA) and then with a goat anti-rabbit horseradish peroxidase conjugate (Sigma). A mouse antibody to α-tubulin (Sigma) was used to monitor sample loading. Specific signals were revealed with chemiluminescence substrates and recorded on films.

Transient transfection and reporter assays. Transient transfection and reporter assays were carried out with 293 cells as previously described (48). About 8 × 10⁵ 293 cells were seeded into each well of 6-well plates 1 day before transfection. Transfection was carried out using Lipofectamine 2000 reagent according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). For transfection with HUVEC, cells were seeded at 2 × 10⁵ cells per well of 6-well plates 1 day before transfection. Transfection of HUVEC was carried out using Cytopure transfection reagent according to the instructions of the manufacturer (MP Biomedicals, Irvine, CA). To further calibrate the transfection efficiency, samples were normalized by cotransfection with a reporter plasmid, pSV-β-galactosidase (Promega, Madison, WI). The cells were collected in 200 μl of 1× lysis buffer (Promega) 36 h after transfection. An aliquot of the supernatant (20 μl) and a lucerase assay system (Promega) were used to measure the lucerase activity, and the results were recorded with a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). An aliquot of the supernatant (20 μl) and a β-galactosidase kit (Promega) were used to measure the β-galactosidase activity. All the reporter assays were independently carried out three times with three repeats each time. Results calculated as the averages with the standard deviations from one representative experiment are presented.

qPCR. Cells were harvested by trypsinization and washed three times with PBS at different time points after KSHV infection and treatment with MAPK inhibitors. DNA from each sample was extracted using a QIAamp DNA blood Mini kit (QIAGEN, Valencia, CA). Quantitative real-time PCR (qPCR) was carried out in a DNA engine Opticon 2 continuous fluorescence detector (Bio-Rad, Hercules, CA) as previously described (52). cDNA primers cDNA-F (CATT GCGCGCCTCTTATATTCA) and cDNA-R (ATGCCGGTGTCGACGCA) were used to detect intracellular KSHV genomic DNA. Primers of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) GAPDH-F (5′AACGC CGCATCCTTCTT) and GAPDH-R (5′ACGACAAATCCGTGACTG) that amplify a product of 94 bp were used to normalize the samples. Each sample was assayed with three repeats.

RT-qPCR. Reverse transcription-qPCR (RT-qPCR) was performed as previously described (53). Briefly, total RNAs from KSHV-infected HUVEC were prepared with TRI reagent as recommended by the manufacturer (Sigma, St. Louis, MO). The RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI) and reverse transcribed to obtain the first-strand cDNA by use of a Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA). A control experiment without reverse transcriptase was conducted in parallel. qPCR was then performed with the cDNA as described above. A primer pair, RTA-F (CACAATAGGGCCCAAGATGA) and RTA-R (TGGTAGAGTTTGGCTTCAAGT), was used to amplify a 98-bp product from the cDNA of all RTA-spliced transcripts. Again, GAPDH was used as a normalization control. Each sample was assayed with three repeats.

RESULTS

Modulation of KSHV infection by multiple MAPK pathways. We have previously shown that KSHV activates all three MAPK pathways, including the JNK, MEK, and p38 pathways, during primary infection (50). To determine whether efficient KSHV infection of cells depends on the activation of MAPK pathways, we infected HUVEC, in the presence of inhibitors of
of these pathways, with a recombinant KSHV BAC36 containing a GFP cassette, which provides a convenient tracking method to monitor KSHV infectivity (56). Cells were pretreated with the inhibitors for 30 min prior to infection and removed 12 h postinfection (hpi). GFP-positive cells were counted at 2 dpi. As shown in Fig. 1A, inhibitors of MAPK pathways JNK inhibitor II, U0126, and SB203580 reduced GFP-positive cell levels by 85%, 50%, and 40%, respectively. As we have previously shown (50), these inhibitors effectively inhibited KSHV activation of JNK, MEK, and p38 pathways, respectively, during primary infection (Fig. 1B). Similar results were observed with 293 cells (Fig. 1C). Since the GFP cassette is exogenous to the KSHV genome, we examined the expression of KSHV latent gene LANA, which is expressed early during primary infection and is necessary for the persistence of KSHV episomes (52, 53). As shown in Fig. 1D, the numbers of LANA-positive cells identified by their speckle nuclear staining were reduced by 82%, 60%, and 36%
in cells treated with inhibitors of JNK, MEK, and p38 pathways, respectively, compared to the results seen with cells that were not treated with the inhibitors. Thus, the reduction of LANA-positive cell numbers by the inhibitors of MAPK pathways was consistent with the results obtained by tracking GFP-positive cells, indicating that GFP was a reliable marker for monitoring KSHV infectivity.

Since successful viral infection should result in the delivery of viral genomes into the cells, we examined the effects of inhibitors of MAPK pathways on the entry of KSHV genomes into the cells. Again, HUVEC were infected with KSHV with or without the presence of inhibitors and quantified for intracellular viral genome copy number per cell by qPCR at 16 hpi. Since there was no evidence of KSHV replication at this time point (19), the detected intracellular viral genomes should represent the viral genomes newly entering the cells during primary infection. As with the results obtained by tracking GFP- and LANA-positive cells, inhibitors of JNK, MEK, and p38 pathways reduced the entry of KSHV genomes into cells by 85%, 76%, and 56%, respectively (Fig. 1E). Together, these results demonstrate that inhibitors of all three MAPK pathways reduce KSHV infectivity.

To determine whether the effects of inhibitors of MAPK pathways on KSHV infectivity were specific, we conducted a dose-response experiment in which different concentrations of the inhibitors were applied during KSHV infection. As shown in Fig. 1F, the number of GFP-positive cells negatively correlated with the concentrations of inhibitors of all three MAPK pathways, thus demonstrating the specific effects of the inhibitors on KSHV infectivity.

KSHV efficient infection of HUVEC is productive at the early stage of infection, during which approximately half of the cells undergo active lytic replication resulting in cell death while the remaining cells establish latency and continue to proliferate (19). It has been reported that KSHV infection of 293 cells induced cytopathic effects (16), suggesting that there might also be a viral lytic replication phase at the early stage of infection in this cell line, though the details of the viral replication program required further investigation. Nevertheless, KSHV eventually establishes latent infection in 293 cells following primary infection (16, 56). To determine the effects of inhibitors of MAPK pathways on KSHV infection over time, we tracked the GFP-positive cells of KSHV-infected cultures with or without the presence of these inhibitors. To facilitate the monitoring of viral infection rate, we infected the cells at a multiplicity of infection of 0.5 to obtain an infection rate of 15 to 30% at 2 dpi. In HUVEC that were treated with inhibitors, GFP-positive cell numbers increased slightly from 2 dpi to 3 dpi but remained stable afterward (Fig. 2A). Similar results were also observed with 293 cells (Fig. 2B). Treatment of HUVEC, 293, or BCBL-1 cells latently infected with BAC36 for a period of 5 days with daily replacement of medium and fresh inhibitors also altered neither the percentage of GFP-positive cells (Fig. 2C) nor the expression of LANA (Fig. 2D). Together, these results indicate that inhibitors of MAPK pathways do not di-
directly affect the expression of GFP or LANA of KSHV-infected cells. Thus, it appears that inhibitors of MAPK pathways have no effect on the establishment and maintenance of viral latency once KSHV has established infection in the cells.

Pharmacological inhibitors often have side effects on the cells. To confirm the roles of MAPK pathways in KSHV infectivity, we applied biological methods to inhibit the MAPK pathways and examined the effects on KSHV infectivity. Cells were transfected with DN constructs of the respective pathways for 12 h before KSHV infection. As shown in Fig. 3A, DN constructs of the JNK, ERK, and p38 pathways reduced KSHV infectivity by 75%, 71%, and 56%, respectively. The results were similar to those obtained with chemical inhibitors (Fig. 1) and clearly indicated the dependence of efficient KSHV infection on the activated MAPK pathways during primary infection.

We further determined whether activation of any of the MAPK pathways was sufficient to enhance KSHV infection. We overexpressed components of JNK, ERK, and p38 pathways and determined their effects on KSHV infectivity. While overexpression of JNK and p38 had minimal effects on KSHV infectivity, overexpression of ERK increased KSHV infectivity by 2.5-fold (Fig. 3B).

Taken together, the above-described results have shown that KSHV infection is modulated by multiple MAPK pathways. Activation of ERK MAPK pathway was necessary and sufficient for efficient KSHV infection whereas activation of JNK and p38 MAPK pathways was necessary but not sufficient for efficient KSHV infection under our experimental conditions.

Inhibitors of MAPK pathways reduced KSHV infectivity at the entry stage of infection. Since inhibitors of MAPK pathways did not directly affect the expression of GFP and LANA (Fig. 2), it was likely that they reduced KSHV infectivity by modulating the early stage of KSHV infection. To identify the stage(s) of KSHV infection that was targeted by inhibitors of MAPK pathways, we quantified KSHV infectivity after applying the inhibitors at different time points postinfection. As shown in Fig. 4A, inhibitors of all three MAPK pathways reduced GFP-positive cell numbers to various extents before 4 hpi but not after 4 hpi. These results indicate that inhibition of KSHV infectivity by the inhibitors indeed occurs at the early stage of infection. We further monitored the kinetics of KSHV virions entering the cells by measuring viral genome copy number per cell at different time points postinfection. DNA extracted from cells infected with KSHV at different time points was subjected to qPCR to determine the intracellular viral genome copy number per cell. As shown in Fig. 4B, KSHV genome copy numbers per cell increased from 0 to 4 hpi; however, after 4 hpi, they remained relatively stable for up to 24 hpi, indicating that the entry of virions and delivery of viral genomes occurred before 4 hpi, and there was an absence of active viral genome duplication during this period. Accordingly, inhibitors of MAPK pathways reduced intracellular viral genome copy numbers only when applied before 4 hpi (Fig. 4C), which was consistent with a reduction of GFP-positive cells when the same inhibitors were applied before 4 hpi (Fig. 4B).
4A). Together, these results indicate that inhibitors of MAPK pathways reduce KSHV infectivity by regulating the entry of virions and delivery of viral genomes into the cells.

Inhibitors of MAPK pathways reduced the production of infectious virions during primary infection of HUVEC. Since efficient KSHV infection of HUVEC is productive at the early stage of infection (19), we determined the effects of inhibitors of MAPK pathways on the production of infectious virions during primary infection. Supernatants from KSHV-infected HUVEC were collected daily at different dpi and replaced with fresh media. Virus titers in the supernatants were measured by infecting 293 cells and calculating the GFUs (19). Production of infectious virions was observed as early as 2 dpi and peaked at 3 dpi (Fig. 5). Inhibitors of all three MAPK pathways significantly reduced the production of infectious virions. Compared to untreated KSHV-infected HUVEC, HUVEC exposed to inhibitors of JNK, MEK, and p38 MAPK pathways produced 93%, 70%, and 73% fewer infectious virions, respectively, at the peak of virion production (Fig. 5).

Inhibitors of MAPK pathways also reduced KSHV productive lytic replication during primary infection at the postentry stage. The reduction of infectious virion numbers by inhibitors of MAPK pathways during primary infection could be due at least in part to their inhibitory effects on KSHV infectivity. Nevertheless, a number of KSHV genes, including RTA, RAP (K8), and MTA (Orf57) (7, 47) as well as lytic origins of DNA replication (oriLyts) (4), are regulated by AP-1. Since KSHV infection activates AP-1 through multiple MAPK pathways (50), it is reasonable to postulate that KSHV productive lytic replication during primary infection could also be directly modulated by the activated MAPK pathways. To test this hypothesis, we added inhibitors of MAPK pathways to HUVEC at 4 hpi since they had minimal effect on KSHV infectivity when added at this time point (Fig. 4A and C). We then determined the titers of the infectious virions in the supernatants of KSHV-infected cells at the peak of the production of KSHV infectious virions (3 dpi). As shown above, addition of inhibitors of JNK, MEK, and p38 MAPK pathways before KSHV infection reduced the production of KSHV infectious virions by 82%, 80%, and 68%, respectively (Fig. 6A). Addition of inhibitors of JNK, MEK, and p38 at 4 hpi also reduced the production of KSHV infectious virions, but to lesser extents, i.e., by 55%, 49% and 25%, respectively (Fig. 6A). These results indicate that besides KSHV infectivity, inhibitors of MAPK pathways have direct inhibitory effects on the production of KSHV infectious virions during primary infection.

KSHV RTA is a master transactivator of viral lytic replication. RTA alone is sufficient and necessary for activating KSHV into full lytic replication (33, 43, 51). We thus determined whether inhibitors of MAPK pathways affected the expression of RTA by measuring the expression level of its transcripts of KSHV-infected cultures at the peak of virion production (3 dpi). The virus was removed after the initial infection by washing the cells three times with culture media. Supernatants were removed from the KSHV-infected cultures at the peak of virion production (3 dpi), and titers were determined for infectious virions. (B) Inhibitors of MAPK pathways reduced the expression of RTA transcripts during primary infection at both early and late stage of infection. HUVEC treated as described for panel A were collected at 48 hpi and analyzed for the expression of RTA transcripts by RT-qPCR. The experiments were carried out two times with three repeats each time. Results represent the averages with standard deviations from one experiment.

FIG. 5. Inhibitors of MAPK reduced the production of KSHV infectious virions during primary infection. HUVEC were infected with KSHV with or without inhibitors of JNK, MEK, and p38 MAPK pathways. The virus was removed after the initial infection by washing the cells three times with culture media. Supernatants were removed daily from the KSHV-infected cultures from 2 to 10 dpi, titers were determined for infectious virions, and the cultures were replaced with fresh medium. The experiments were carried out two times with three repeats each time. Results represent the averages with standard deviations from one experiment.

FIG. 6. Inhibition of MAPK pathways blocks KSHV productive replication at both entry and postentry stages during KSHV primary infection. (A) Inhibitors of MAPK pathways reduced the production of KSHV infectious virions during primary infection at both early and late stages of infection. Inhibitors of JNK, MEK, and p38 MAPK pathways were added to KSHV-infected HUVEC at 0 hpi and 4 hpi. The virus was removed after the initial infection by washing the cells three times with culture media. Supernatants were removed from the KSHV-infected cultures at the peak of virion production (3 dpi), and titers were determined for infectious virions. (B) Inhibitors of MAPK pathways reduced the expression of RTA transcripts during primary infection at both early and late stage of infection. HUVEC treated as described for panel A were collected at 48 hpi and analyzed for the expression of RTA transcripts by RT-qPCR. The experiments were carried out two times with three repeats each time. Results represent the averages with standard deviations from one experiment.
scripts by RT-qPCR. In consistency with the production of KSHV infectious virions, addition of inhibitors of JNK, MEK, and p38 pathways at the time of KSHV infection reduced the expression of RTA transcripts by 78%, 61%, and 41%, respectively, while addition of the inhibitors at 4 hpi also reduced the expression of RTA transcripts, but to lesser extents, i.e., by 49%, 38%, and 22%, respectively (Fig. 6B). These results indicate that multiple MAPK pathways regulate RTA expression not only at the entry stage of infection but also at the postentry stage of infection.

Multiple MAPK pathways modulate RTA promoter activity during primary infection. We have shown that KSHV infection activates multiple MAPK pathways early during primary infection preceding the expression of RTA transcripts, which only starts to increase at significant levels after 6 hpi (50, 53). Thus, it is likely that the regulation of expression of RTA transcripts by MAPK pathways is at the transcriptional level. We first investigated whether KSHV activated the RTA promoter during primary infection in a reporter assay. A full-length RTA promoter reporter construct and assayed for luciferase activity at 36 h posttransfection. Prior to harvest, the cells were infected with KSHV for 0, 6, 12, and 24 h. (B) Inhibitors of MAPK pathways inhibited KSHV activation of RTA promoter during primary infection. HUVEC and 293 cells transfected with the RTA promoter reporter construct for 30 h were either mock infected or infected with KSHV for 6 h with or without inhibitors of JNK, MEK, and p38, lysed, and assayed for luciferase activity. (C) DN constructs of MAPK pathways inhibited KSHV activation of the RTA promoter during primary infection. HUVEC and 293 cells transfected with the RTA promoter reporter construct together with a vector control (V) or DN constructs of JNK, MEK, and p38 MAPK pathways for 30 h were either mock infected or infected with KSHV for 6 h, lysed, and assayed for luciferase activity. (D) Inhibitors of MAPK pathways inhibited KSHV activation of the RTA promoter at both early and late stages of infection. HUVEC and 293 cells transfected with the RTA promoter reporter construct for 30 h were either mock infected or infected with KSHV for 6 h with or without inhibitors of JNK, MEK, and p38 MAPK pathways, lysed, and assayed for luciferase activity. Inhibitors were added at either 0 hpi or 4 hpi. (E) UV-irradiated KSHV virions retained the ability to activate the RTA promoter. HUVEC and 293 cells were transfected with the RTA promoter reporter construct and assayed for luciferase activity at 36 h posttransfection. At 6 h prior to harvest, the cells were mock infected or infected with KSHV or UV-irradiated KSHV. UV irradiation reduced the virus infectivity from 70% to 80% to less than 1%. All the experiments were independently carried out three times except those with HUVEC, which were carried out two times with three repeats each time. Results represent the averages with standard deviations from one representative experiment.
during primary infection by inhibitors of MAPK pathways could be due to their inhibitory effects on KSHV infectivity (Fig. 1). Nevertheless, the RTA promoter activity could also be independently modulated by the activated MAPK pathways during KSHV primary infection. Therefore, we examined the effects of inhibitors of MAPK pathways on KSHV activation of the RTA promoter by adding them at 4 hpi. As shown in Fig. 7D, inhibitors of all three MAPK pathways reduced KSHV activation of the RTA reporter when they were added at 4 hpi, albeit to lesser extents than when they were added at the time of infection (0 hpi). These results are consistent with those obtained with RTA transcripts (Fig. 6B) and indicate that the MAPK pathways directly modulate the RTA promoter during KSHV primary infection and that these effects are independent of their regulation of KSHV infectivity.

The activation of MAPK pathways during KSHV primary infection occurs at the early stage of infection (<1 hpi), probably as the result of interactions between viral ligands and cellular receptors (50). Thus, the RTA promoter could be directly modulated by MAPK pathways activated during the KSHV entry stage of infection. Indeed, UV-irradiated KSHV virions could still activate the RTA promoter (Fig. 7E), which is in agreement with the results of a recent study (32).

MAPK pathways mediate KSHV activation of the RTA promoter through AP-1 during primary infection. The fact that an AP-1 consensus-binding site is present in the RTA promoter suggests that it might be the pathway through which MAPK pathways mediate its activation during KSHV primary infection. This is consistent with our observation of AP-1 activation by multiple MAPK pathways during KSHV primary infection (50). Indeed, DN constructs of c-Fos and c-Jun, the two major components of AP-1, inhibited KSHV activation of the RTA reporter (Fig. 8A and B). As expected, overexpression of c-Fos or c-Jun alone was sufficient to activate the RTA promoter, which was inhibited by cotransfection of their respective DN constructs (Fig. 8C and D). These results illustrate the direct involvement of AP-1 in KSHV activation of the RTA promoter during primary infection.

To further confirm the role of AP-1 in the activation of RTA promoter during KSHV primary infection and identify the cis element(s) in the RTA promoter that was responsive to KSHV infection, we performed reporter assays with a series of deletion reporter constructs of the RTA promoter (Fig. 9A). As shown in Fig. 9B, the dominant KSHV-responsive region was located between −15 and −259 in the RTA promoter. Analysis of this DNA fragment identified a conserved AP-1 site between −81 and −87, which has previously been shown to be functional in response to O-tetradecanoylphorbol 13-acetate induction (47). To determine whether this AP-1 site was indeed the major site responsive to KSHV infection, we carried out site-directed mutagenesis using the R-259 reporter construct as a template and generated R-259mut, a corresponding reporter construct with the AP-1 site ablated (Fig. 9C). As shown in Fig. 9D, mutation of the AP-1 site completely abolished the responsiveness of the RTA reporter to KSHV infection. As expected, overexpression of c-Fos or c-Jun alone was sufficient to activate the R-259 reporter, which was inhibited by cotransfection of their respective DN constructs, whereas over-
expression of c-Fos or c-Jun failed to activate the R-259mut reporter (Fig. 9E). These results illustrate the direct involvement of AP-1 in KSHV activation of the RTA promoter during primary infection. These results further indicate that the AP-1 site is indeed the dominant site responding to KSHV during primary infection and thus further confirm an essential role of the AP-1 pathway in the activation of RTA promoter during KSHV primary infection.

**DISCUSSION**

Activation of cellular signaling pathways is a common phenomenon during infection by many viruses. Successful viral infection often depends on the modulation of these cellular pathways. For example, human immunodeficiency virus type 1 depends on the activation of MEK MAPK pathway to deliver its genomes into CD4+ cells (31). Similarly, it has been reported that the MEK MAPK pathway plays an important role in KSHV infection. KSHV activates MEK MAPK pathway at the early stage of primary infection, and the MEK pathway, in turn facilitates KSHV infection at the postattachment stage (1, 38, 50). Specific inhibitors of MEK MAPK pathway reduce KSHV infectivity, whereas constitutive expression of the upstream kinase Raf enhances KSHV infectivity (1, 26).

In this report, we have also described modulation of KSHV infection by the MEK MAPK pathway. Based on our previous observation that KSHV activates JNK, MEK, and p38 MAPK pathways during primary infection (50), we present further evidence to show that the JNK and p38 MAPK pathways are also involved in KSHV infection. Inhibition of all three MAPK pathways resulted in significant reduction of KSHV infectivity (Fig. 1 and 3), indicating that activation of these pathways is
necessary for efficient KSHV infection. We have also shown that activation of MEK pathway alone led to enhanced KSHV infection (Fig. 3B), which was consistent with the results of previous reports (1, 26). In contrast, activation of JNK and p38 pathways did not lead to enhanced KSHV infectivity, indicating that activation of these two pathways, though necessary, is not sufficient to promote KSHV infection. KSHV enters the host cells through clathrin-mediated endocytosis (2), a complicated process that includes binding of ligand(s) to the receptor(s) and subsequent internalization. Inhibition of MEK/ERK and upstream phosphatidylinositol-3 kinase/protein kinase C delta (PKCδ) pathways reduces KSHV infectivity but has no effect on virus binding to the cell surface receptor (38), implicating the MEK MAPK pathway in the postattachment stage in processes such as internalization. It is therefore reasonable to assume similar roles for the JNK and p38 MAPK pathways in KSHV infection. Indeed, we have shown that inhibition of KSHV infectivity by inhibitors of MAPK pathways occurs during the virus entry process (Fig. 4).

Cytoskeleton assembly activated by various kinases plays an important role in the endocytosis of many viruses (24). For example, activation of actin cytoskeleton by the GTPase of the Rho family mediates the entry of adenovirus into the host cells (35, 36) whereas actin cytoskeleton reorganization induced by Rac is required for influenza virus entry into the epithelial cells (46). The interaction of the RGD motif within KSHV gB with integrin α3β1 activates focal adhesion kinase and the downstream phosphatidylinositol-3 kinase/PKCδ and induces cytoskeleton assembly processes such as polymerization of cortical actin filaments (38). Two functions have been associated with the polymerization of actin filaments. First, it might provide a force to form the endocytic vesicles and sever them from the cell membrane as well as stabilize the membrane integrin molecules by acting as a platform (8, 13, 36), which could facilitate the virus-host interactions. Second, it provides a force for the trafficking of the engulfed vesicles within the cytoplasm at a later stage of endocytosis (15). Therefore, we could speculate that the MEK, JNK, and p38 MAPK pathways could also regulate KSHV entry by modulating similar cytoskeleton pathways. It has been demonstrated that activated ERK1/2 can phosphorylate neurofilaments and paxillin, both of which are important cytoskeleton proteins (10). In addition, cytoskeleton-associated protein filament A is phosphorylated by RSK1, a downstream kinase of MEK pathway (49), indicating the role of MEK pathway in cytoskeleton reorganization. Similarly, p38 has been shown to phosphorylate microtubule-associated protein Tau (30) and the JNK pathway activates AP-1 components to regulate cytoskeleton organizations (34). In addition to direct targeting of cytoskeletal proteins, KSHV-activated MAPK pathways could modulate the expression and modification of many other cellular proteins to facilitate viral infection.

The activation of PKC and MEK is also involved in virus postentry stages, such as transport of virus to the nucleus and delivery of viral genomes into the nucleus (38). The activation of JNK and p38 pathways could also be involved in the same processes. On the other hand, although these pathways might be involved in the late stage of infection, the reduction of infectivity caused by MAPK inhibition is more likely due to the defect in the early stage of internalization, since such inhibition resulted in the reduced entry of KSHV genomes into the cells (Fig. 4). Once the viral genomes are delivered into the cells, they remain relatively stable for up to 24 hpi (Fig. 4B). Thus, the reduced KSHV genomes in the cells resulting from inhibition of MAPK pathways are a direct effect on virus entry into the cells but are not due to any enhanced degradation of viral genomes in an unfavorable intracellular environment.

In addition to their roles in KSHV entry, we have found that JNK, MEK, and p38 pathways are involved at least in part in viral productive lytic replication by upregulating the expression of RTA. Our previous studies have revealed that a number of viral lytic genes, including RTA, are expressed at the early stage of infection (53). RTA alone can initiate the lytic replication cascade through transactivation of other viral lytic genes and the initiation of lytic replication through binding to OriLyt (4, 25, 33, 43, 51). We have recently found that MAPK pathways mediate the activation of AP-1 complex during KSHV primary infection (50). As is consistent with the presence of an AP-1 consensus site in the promoter region of RTA (47), we have demonstrated that KSHV activation of RTA promoter is accomplished predominantly via the AP-1 pathway (Fig. 8 and 9). Since the OriLyt also contains several AP-1 sites in the promoter region of RTA (4), we can envisage that KSHV can also activate OriLyt via the AP-1 pathway during primary infection. Our data support the idea that there is at least one (likely the major if not the sole) mechanism by which the lytic replication is regulated during primary infection. Since KSHV reactivation from latency depends on the expression of RTA, it can be postulated that this process is also mediated by multiple MAPK pathways.

We have shown that UV-irradiated KSHV virions are capable of activating as much as 50% to 70% of the RTA promoter activity (Fig. 7E), indicating that the interaction between KSHV ligand(s) and receptor(s) alone significantly contributes to this process. It would be interesting to determine what other KSHV ligand(s) and cellular receptor(s), if they are not gB and α3β1, mediate the activation of JNK and p38 pathways during primary infection. It remains possible that the encapsidated virion proteins and transcripts could also lead to the activation of MAPK pathways during KSHV primary infection (5, 6, 53). It is intriguing that only a subset of the cells undergo viral lytic replication while the rest of them enter viral latency during KSHV primary infection (19). It can be speculated that KSHV infection could lead to a differential activation of the MAPK pathways in the individual cells, particularly when they were at different growth or cell cycle phases. Such differential activation of cellular signaling pathways could certainly lead to divergent directions in viral life cycles.

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REFERENCES


29. Thomas, B., S. Thirion, L. Humbert, L. Tan, M. B. Goldring, G. Bereziat,


