ERK1/2 and MEK1/2 Induced by Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Early during Infection of Target Cells Are Essential for Expression of Viral Genes and for Establishment of Infection

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Kaposi's sarcoma-associated herpesvirus (KSHV) in vitro target cell infection is characterized by the expression of the latency-associated genes ORF 73 (LANA-1), ORF 72, and K13 and by the transient expression of a very limited number of lytic genes such as lytic cycle switch gene ORF 50 (RTA) and the immediate early (IE) lytic K5, K8, and v-IRF2 genes. During the early stages of infection, several overlapping multistep complex events precede the initiation of viral gene expression. KSHV envelope glycoprotein gB induces the FAK-Src-PI3K-RhoGTPase (where FAK is focal adhesion kinase) signaling pathway. As early as 5 min postinfection (p.i.), KSHV induced the extracellular signal-regulated kinase 1 and 2 (ERK1/2) via the PI3K-PKC ζ -MEK pathway. In addition, KSHV modulated the transcription of several host genes of primary human dermal microvascular endothelial cells (HMVEC-d) and fibroblast (HFF) cells by 2 h and 4 h p.i. Neutralization of virus entry and infection by PI-3K and other cellular tyrosine kinase inhibitors suggested a critical role for signaling molecules in KSHV infection of target cells. Here we investigated the induction of ERK1/2 by KSHV and KSHV envelope glycoproteins gB and gpK8.1A and the role of induced ERK in viral and host gene expression. Early during infection, significant ERK1/2 induction was observed even with low multiplicity of infection of live and UV-inactivated KSHV in serum-starved cells as well as in the presence of serum. Entry of UV-inactivated virus and the absence of viral gene expression suggested that ERK1/2 induction is mediated by the initial signal cascade induced by KSHV binding and entry. Purified soluble gpK8.1A induced the MEK1/2 dependent ERK1/2 but not ERK5 and p38 mitogen-activated protein kinase (MAPK) in HMVEC-d and HFF. Moderate ERK induction with soluble gB was seen only in HMVEC-d. Preincubation of gpK8.1A with heparin or anti-gpK8.1A antibodies inhibited the ERK induction. U0126, a selective inhibitor for MEK/ERK blocked the gpK8.1A- and KSHV-induced ERK activation. ERK1/2 inhibition did not block viral DNA internalization and had no significant effect on nuclear delivery of KSHV DNA during de novo infection. Analyses of viral gene expression by quantitative real-time reverse transcriptase PCR revealed that pretreatment of cells with U0126 for 1 h and during the 2-h infection with KSHV significantly inhibited the expression of ORF 73, ORF 50 (RTA), and the IE-K8 and v-IRF2 genes. However, the expression of lytic IE-K5 gene was not affected significantly. Expression of ORF 73 in BCBL-1 cells was also significantly inhibited by preincubation with U0126. Inhibition of ERK1/2 also inhibited the transcription of some of the vital host genes such as DUSP5 (dual specificity phosphatase 5), ICAM-1 (intercellular adhesion molecule 1), heparin binding epidermal growth factor, and vascular endothelial growth factor that were up-regulated early during KSHV infection. Several MAPK-regulated host transcription factors such as c-Jun, STAT1 α , MEK2, c-Myc, ATF-2 and c-Fos were induced early during infection, and ERK inhibition significantly blocked the c-Fos, c-Jun, c-Myc, and STAT1 α activation in the infected cells. AP1 transcription factors binding to the RTA promoter in electrophoretic mobility shift assays were readily detected in the infected cell nuclear extracts which were significantly reduced by ERK inhibition. Together, these results suggest that very early during de novo infection, KSHV induces the ERK1/2 to modulate the initiation of viral gene expression and host cell genes, which further supports our hypothesis that beside the conduit for viral DNA delivery into the cytoplasm, KSHV interactions with host cell receptor(s) create an appropriate intracellular environment facilitating infection.

Kaposi's sarcoma-associated herpesvirus (KSHV), a member of the lymphotropic human gamma-2 herpesvirus family (genus *Rhadinovirus*) (45, 50) is etiologically linked with Ka-

posi's sarcoma (KS), a multifocal endothelial cell tumor most commonly seen in AIDS patients (14). KS lesions are characterized by the presence of spindle-shaped endothelial cells and inflammatory cells. Several lines of evidence point to a central role of KSHV in the pathogenesis of KS and in the pathogenesis of two B cell-proliferative disorders, primary effusion lymphoma or body cavity-based B-cell lymphomas (BCBL) and multicentric Castleman's disease (53). Cell lines with B-cell characteristics established from BCBL carry KSHV in a latent

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form, and a lytic cycle can be induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

KSHV DNA sequence analyses demonstrate that a large region of the KSHV genome is conserved among herpesviruses and is colinear with gamma-1 Epstein-Barr virus and gamma-2 herpesvirus saimiri (46, 50). KSHV encodes more than 90 open reading frames (ORFs), which are designated as ORFs 4 to 75 by their homology to herpesvirus saimiri ORFs (46, 50). Divergent regions in between the conserved gene blocks contain more than 20 KSHV unique genes, which are designated with the prefix K. Several KSHV-encoded proteins are homologs of host proteins. These genes include K2 (v-interleukin-6), K4 (v-macrophage inhibitory protein II), K3 and K5 (MIR-1 and MIR-2; immunomodulatory proteins), K6 (v-macrophage inhibitory protein I), K7 (antiapoptotic protein), K9 (v-interferon regulatory factor [vIRF]), vIRF2 (K11.1), ORF 16 (vBcl-2), K13 (v-FLICE-inhibitory protein), K14 (vOX-2), ORF 72 (v-cyclin D), and ORF 74 (v-G protein-coupled receptor) (17, 21, 22, 40, 46, 50, 52, 53).

KSHV DNA and transcripts have been identified *in vivo* in KS spindle cells, human B cells, endothelial cells, keratinocytes, prostate epithelial cells, B cells, and macrophages (8, 11, 16, 53, 69). KSHV infects a variety of cell types *in vitro*, which include human B, endothelial, epithelial, fibroblast cells, keratinocytes, owl monkey kidney cells, baby hamster kidney (BHK-21) cells, Chinese hamster ovary (CHO) cells, and primary embryonic mouse fibroblast cells (4, 9, 44, 49, 52, 62). Infection is characterized by the expression of latency-associated ORF 73, ORF 72, and K13 genes and the transient expression of a very limited number of lytic genes such as ORF 50, K5, K8, and v-IRF2 (17, 32).

Like other herpesviruses, KSHV encodes a number of envelope-associated glycoproteins, and KSHV glycoproteins gB (ORF 8), gH (ORF 22), gM (ORF 39), gL (ORF 47), and gN (ORF 53) are counterparts to other herpesvirus glycoproteins (45, 50). In addition to these conserved glycoproteins, KSHV also encodes K1, gpK8.1A, gpK8.1B, and K14, which are unique to KSHV (13, 45, 50). Our previous studies showed that KSHV uses cell surface heparan sulfate (HS)-like molecules to bind target cells. We along with others have also demonstrated the interaction of virion envelope-associated KSHV glycoprotein gB and gpK8.1A with HS molecules (2, 3, 65). KSHV gB possesses the integrin binding RGD motif, and we have demonstrated the inhibition of KSHV infectivity by antibodies against $\alpha 3$ and $\beta 1$ integrins and soluble $\alpha 3\beta 1$ integrin (4). Anti-KSHV-gB antibodies immunoprecipitated the virus- $\alpha 3\beta 1$ complex. Radiolabeled virus binding studies suggest that KSHV uses the $\alpha 3\beta 1$ integrin as one of the cellular receptors for entry into target cells. Using an RTA-dependent reporter 293 T-cell line, Inoue et al. (23) reported the inability of soluble $\alpha 3\beta 1$ integrin and RGD peptides to block the infectivity of KSHV. However, in this study virus was centrifuged with cells in the presence of polybrene, which may account for the apparent discrepancy. Polybrene is a positively charged cation which complexes with the virus envelope and bypasses the need for receptors (33). This property of polybrene is the basis for its use to increase the infectivity of many viruses and to deliver nucleic acid for gene therapy (33). The nature of other recep-

tor(s) recognized by KSHV and the glycoproteins involved need to be evaluated further.

Using soluble gB protein, we have demonstrated the ability of gB to mediate extensive cytoskeletal rearrangement in the target cells via a FAK-Src-PI3-kinase-Rho-GTPases signaling pathway (54). As early as 5 min postinfection (p.i.), KSHV induced the extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) superfamily via PI3K-PKC ζ -MEK pathway (44). Inhibitors specific for phosphatidylinositol (PI) 3-kinase, protein kinase C ζ (PKC ζ), MEK, and ERK1/2 significantly reduced the infectivity of KSHV without affecting its binding to the target cells. Examination of viral DNA entry suggests a role for PI 3-kinase in KSHV entry into the target cells and a role for PKC ζ , MEK, and ERK at a postviral entry stage of infection (44, 54). The role of KSHV envelope glycoproteins in initiating the ERK1/2 induction was unknown.

Here we present several lines of evidences to show that ERK1/2 is induced by KSHV gpK8.1A and gB and that this induction plays a major role in KSHV gene expression. These findings implicate a critical role for KSHV-induced signaling in its infection of target cells and suggest that, by orchestrating the signal cascade, KSHV may create an appropriate intracellular environment to facilitate the infection.

MATERIALS AND METHODS

Cells. Human foreskin fibroblast (HFF) cells (Clonetics, Walkersville, Md.) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, N.Y.) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, Utah), 2 mM L-glutamine, and antibiotics. Human microvascular dermal endothelial cells (HMVEC-d) (CC-2543; Clonetics) were grown in endothelial basal media-2 and growth factors (Clonetics). Recombinant green fluorescent protein-KSHV (GFP-KSHV- γ KSHV.152) carrying BCBL-1 cells (62) were cultured in RPMI 1640 (Gibco BRL) medium (44). All cells were cultured in lipopolysaccharide (LPS)-free medium.

Antibodies, substrates, and chemicals. Polyclonal rabbit antibodies immunoprecipitating ERK2 (sc-154) and MEK (sc-219) as active kinases were from Santa Cruz Biotechnology, Inc., Santa Cruz, Calif. Rabbit antibodies detecting the phosphorylated forms of ERK1/2 (Thr 202/Tyr 204 phospho-p44/42 MAP kinase), MEK1/2 (Ser 217/221 phospho-MEK1/2), p38 MAP kinase (Thr180/Tyr182 phospho-p38 MAPK) antibodies, and PD98059 (MEK1 inhibitor) were from Cell Signaling Technology, Beverly, Mass. Monoclonal antibodies detecting phosphotyrosine (PY20) and mouse anti-phospho-FAK antibody (Y397) were from Transduction Laboratories, Los Angeles, Calif. Antirabbit and anti-mouse horseradish peroxidase-, fluorescein isothiocyanate-, or tetramethyl rhodamine isothiocyanate-linked antibodies were from KPL, Inc., Gaithersburg, Md. Rabbit (polyclonal) anti-ERK5/BMK1 (pTpY^{218/220}) phospho-specific antibody was from Biosource International, Calif. Rabbit (polyclonal) anti-ERK5/BMK1 antibody was from StressGen Biotechnologies Corp., British Columbia Canada. Monoclonal (MAb) antibodies against MAP kinase (anti-diphosphorylated ERK1/2 MAPK-YT), α -tubulin, anti- β tubulin 1, and β -actin (clone AC-40) were from Sigma, St. Louis, Mo. NuSieve GTG agarose was from Cambrex Bio Science Rockland, Inc., Maine. Lysophosphatidic acid, LY294002 [20(4-morphodiny)-8-phenyl-1(4H)-benzopyran-4-one], sorbitol, heparin, sodium orthovanadate, benzamide, leupeptin, aprotinin, and epidermal growth factor (EGF) were obtained from Sigma. The ERK substrate, myelin basic protein (MBP), was from Upstate Biotechnology, Lake Placid, N.Y. U0126 (1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio]butadiene), U0124 (1,4-diamino-2,3-dicyano-1,4-bis [methylthio]butadiene), *Clostridium difficile* toxin A (CdTxA), and MAb against lamin B were from Calbiochem, La Jolla, Calif. Protein A Sepharose CL-4B beads were from Amersham Pharmacia Biotech, Piscataway, N.J. Polyclonal rabbit antibody for total p38 was from Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.

Recombinant KSHV proteins. The generation and purification of recombinant baculovirus-expressed Δ TMgB, Δ TMgB-RGA, Δ TMgpK8.1A, and ORF 73 proteins have been previously described (64, 65, 74). Recombinant protein purifications were carried out in buffers prepared with LPS-free water, and stock

TABLE 1. Primer and probe sequences used for real-time PCR

Gene	K5	K8	v-IRF2
Forward primer	5'-GAGCGTCCAGGTGCAAC-3'	5'-CCTGGACGCTCTCTCACACA-3'	5'-CATTTTGGAGGAGCGACGTA-3'
Reverse primer	5'-TTGAACGTGTTCTGCTGATGCTG-3'	5'-GGATCTGCGAGTTGGAAGCT-3'	5'-AGCCCAGGCCAGTCTCAGT-3'
TaqMan probe	6FAMACGCCGACAAGCCAGCCAC TAMRA	6FAMCCAAGAGGACCACACATTTTCGCA TAMRA	6FAMCGGGCTGCCAGAAATC CCGG TAMRA
Product length	66 bp	71 bp	74 bp

preparations of proteins were monitored for endotoxin contaminations by standard *Limulus* assay (*Limulus* Amebocyte Lysate Endochrome; Charles River Endosafe, Charleston, S.C.) as recommended by the manufacturer.

Virus. Induction of the KSHV lytic cycle in GFP-BCBL-1 cells, supernatant collection, and virus purification procedures were described previously (44), and purity was assessed by general guidelines established in our laboratory (4, 32, 44). KSHV DNA was extracted from the virus, and the copy numbers were quantitated by real-time DNA PCR using primers amplifying the KSHV ORF 73 gene as described previously (32). To prepare replication-defective virus, KSHV was inactivated with UV light (365 nm) for 20 min at a 10-cm distance.

KSHV antibodies. The generation of rabbit polyclonal antibodies against KSHV Δ TMgB and MAbs against gpK8.1A has been described previously (64, 74).

Cytotoxicity assay. Target cells were tested for their viability in the presence of various concentrations of soluble recombinant proteins or various inhibitors at 37°C using a lactate dehydrogenase cytotoxicity assay kit (Promega, Madison, Wis.) as described previously (54, 64).

Western blotting. Target cells grown to confluence in 25-cm² flasks were serum starved for 24 h, cooled to 4°C, and induced with KSHV proteins or ligands at 37°C. Cells were exposed to kinase inhibitors (U0126 and LY294002) for 1 h at 37°C prior to KSHV infection or protein induction. As a control, cells were treated with U0124 (Calbiochem), a chemical analogue of U0126 without MEK-inhibitory activity (19). After treatment, cells were washed twice with phosphate-buffered saline (PBS), and total protein was extracted. In some experiments, Δ TMgB and Δ TMgpK8.1A were incubated with anti- Δ TMgB and anti- Δ TMgpK8.1A antibodies for 1 h at 37°C before being added to the cells. Total cell lysates (15 μ g) were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to nitrocellulose membranes, and immunoblotted with antibodies. Immunoreactive bands were developed by enhanced chemiluminescence reaction (NEN Life Sciences Products, Boston, Mass.) and quantified following standard protocols (54).

Immunoprecipitation. For immunoprecipitation reactions, 500 μ g of whole-cell extract was mixed with respective antibodies and rocked overnight at 4°C. A total of 100 μ l of protein A-Sepharose was then added, and rocking was allowed to continue for another 90 min. Beads were then washed four times with RIPA buffer. Fifty microliters of 2 $\times$ SDS sample buffer was added to the beads and boiled for 4 min. The insoluble material was removed by centrifugation, and the supernatant was then subjected to Western blot analysis. The membranes were soaked in blocking solution (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5% bovine serum albumin, 0.02% Na₂S₂O₄) at 4°C overnight and then reacted with phospho-specific antibodies overnight at 4°C. The membranes were washed five times with washing buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.3% Tween 20) and probed with secondary antibodies conjugated with horseradish peroxidase or alkaline phosphatase for 1 h at room temperature. These were detected by using either enhanced chemiluminescence as specified by manufacturer (NEN) or CDP-Star (Roche Diagnostics Corp., Indianapolis, Ind.). The bands were scanned and the band intensities were assessed using the ImageQuant software program (Molecular Dynamics, Sunnyvale, Calif.).

Immune complex kinase assays. Immune complexes were washed five times with lysis buffer, twice with kinase buffer (10 mM MgCl₂, 25 mM β -glycerophosphate, 25 mM HEPES, pH 7.5, 5 mM benzamidine, 0.5 mM dithiothreitol, 1 mM Na₃VO₄). Kinase reactions were initiated by the addition of 2 μ Ci of [γ -³²P]ATP (Perkin Elmer, Boston, MA) and incubated with 5 μ g of substrate (MBP for ERK) in the kinase buffer for 20 min at 30°C. Reactions were terminated by adding 2 $\times$ SDS sample buffer, boiled for 5 min, and analyzed by SDS-12% PAGE. Gels were dried, and phosphorylated substrate was detected by phosphorimager analyses and by autoradiography. Bands were scanned and the band intensities were assessed.

Immunofluorescence assay. Confluent HFF cells in eight-well chamber slides (Nalge Nunc International, Naperville, Ill.) were incubated with either KSHV or 10% FBS for 30 min at 37°C. For phosphorylated p42/p44 MAPKs staining, cells

were fixed with 10% paraformaldehyde and permeabilized with methanol. For total p42/p44 MAPKs staining, methanol-acetone (70:30, vol/vol) at -20°C for fixation and permeabilization was used. Alexa 594-coupled anti-mouse antibodies (Molecular Probes, Eugene, OR) and Alexa 488-coupled anti-rabbit antibodies (Molecular Probes) were used at a 1:250 dilution to detect the phosphorylated p42/p44 MAPK and total p42/p44 MAPK, respectively. Stained cells were washed and viewed with appropriate filters under a fluorescence microscope with the Nikon Magna Firewire digital imaging system.

Preparation of DNA and RNA. Total DNA from the viral stocks and cells were prepared using a DNeasy Tissue kit (QIAGEN, Inc., Valencia, Calif.). Monolayers of infected cells were trypsinized for 5 min at 37°C and collected with 10 ml of ice-cold DMEM. Cells were pelleted at 1,000 rpm for 10 min, washed, and resuspended in 200 μ l of 1 $\times$ PBS, and total DNA was prepared according to the manufacturer's instructions. For the preparation of DNA from intact virions, 200 μ l of virus stocks was pretreated with 12 μ g of DNase I for 15 min at room temperature; the reaction was stopped by EDTA followed by heat inactivation at 70°C, and DNA was prepared. Total RNA was isolated from infected or uninfected cells using an RNeasy kit as described previously (32).

Measurement of KSHV internalization by real-time DNA PCR. Untreated HFF cells or HFF cells incubated with inhibitors were infected with KSHV at 10 DNA copies/cell. After a 2-h incubation, cells were washed twice with PBS to remove the unbound virus, treated with trypsin-EDTA for 5 min at 37°C to remove the bound but noninternalized virus, and washed, and total DNA was isolated using a DNeasy kit. A total of 100 ng of DNA samples, KSHV ORF 73 gene TaqMan probe (32), and Quantitect PCR mix were used. The KSHV ORF 73 gene cloned in the pGEM-T vector (Promega) was used for the external standard. Known amounts of ORF 73 plasmid were used in the amplification reactions along with the test samples. The lower limit of ORF 73 gene detection was 10 to 100 copies, and the most accurate detection was from 100 to 10⁶ copies. Special care was taken to keep the slope of the standard curve close to 3.3 so that the amplification efficiency of the cycles was 2. The cycle threshold (C_T) values were used to plot the standard graph and to calculate the relative copy numbers of viral DNA in the samples.

DNA nuclear delivery assay. Pure nuclear fractions were prepared using a Nuclei EZ isolation kit (Sigma) following the manufacturer's instructions. Cells were pretreated with either 300 ng/ml of CdTxA or 10 μ M U0126, U0124, or 50 μ M LY294002 for 1 h at 37°C. Cells were infected with KSHV for 2 h, washed, treated with trypsin-EDTA to remove noninternalized virus, and lysed on ice for 5 min with a mild lysis buffer (Sigma), and nuclei were concentrated by centrifugation at 500 $\times$ g for 5 min. Cytoskeletal components loosely bound to the nuclei were removed from the nuclear pellet by a repeat of lysis and centrifugation procedures as described previously (42). The nuclei were purified and assessed for their purity by immunoblotting with anti-lamin B antibodies, and cytoskeletal contamination was ruled out by immunoblotting with anti- β -actin and anti- α -tubulin antibodies.

Semiquantitative RT-PCR. Expression kinetics of host genes were analyzed by reverse transcriptase PCR (RT-PCR) using procedures described previously (43). DNase-treated total RNAs were subjected to RT-PCR using specific primers. Successive samples were removed from every three cycles (14 to 41), resolved on agarose gel, and increases in expression (n -fold) were calculated after normalizing to the β -actin gene. For all genes, integrated density values (IDV) corresponding to the sum of pixel intensities after background corrections were recorded for both the KSHV-infected and U0126-pretreated samples at linear points on the amplification curve and changes in expression were calculated after normalizing to β -actin gene.

Real-time RT-PCR. The ORF 50 and ORF 73 transcripts were detected by real-time RT-PCR using specific real-time primers and specific TaqMan probes as described previously (32). K5, K8, and v-IRF2 transcripts were detected using the gene-specific real-time PCR primers and specific TaqMan probes (Applied Biosystems, Foster City, Calif.) described in Table 1. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the internal control. The reaction

conditions used for the ORF 50 and 73 gene amplifications consisted of four stages: 50°C for 2 min, 60°C for 30 min, and 95°C for 10 min, followed by 44 cycles of 95°C for 15 s and 60°C for 30 s. K5, K8, and v-IRF2 gene amplifications consisted of four stages: 50°C for 2 min, 60°C for 30 min, and 95°C for 10 min, followed by 44 cycles of 95°C for 20 s and 55°C for 30 sec. GAPDH conditions varied slightly in the amplification stage, with denaturation of the strands at 95°C for 20 s and 62°C for 1 min, and were repeated for 40 cycles.

Preparation of nuclear extracts. HFF cells were pretreated with 10 μ M U0126 at 37°C for 1 h, infected with KSHV (multiplicity of infection [MOI] of 10) for 5, 15, 30, 60, and 120 min, and nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif Corp, Carlsbad, CA) per the manufacturer's instructions. After measuring protein concentrations by bicinchoninic acid protein assay reagent (Pierce Biotechnology, Rockford, IL), extracts were stored at -70°C. Purity of the nuclear extracts was assessed by immunoblotting using anti-lamin B antibodies, and cytoskeletal contamination was checked by using anti- β -actin and anti- β tubulin 1 antibodies (Sigma).

Transcription factor activation assay. A total of 5 μ g of each nuclear extract was used for this assay. Transcription factors ATF-2, c-Jun, c-Myc, c-Fos, MEF2, and STAT1 α in the nuclear extracts were measured using an enzyme-linked immunosorbent assay (ELISA)-based TransAM MAPK Family kit (Active Motif Corp.) per the manufacturer's instructions. In this assay, transcription factors bind to the immobilized oligonucleotide containing the consensus sequences specific for the particular transcription factor, which is detected by a sandwich ELISA. The active form of the transcription factor contained in the nuclear extract specifically binds to this oligonucleotide mixture. The primary antibodies used to detect each of the MAPK-regulated transcription factors recognize an epitope in the phosphorylated ATF-2, c-Jun, c-Myc, MEF2, c-Fos, and STAT1 α that is accessible only when these transcription factors are activated and bound to their target DNA. The detection limit for the TransAM MAPK Family kit is <0.5 μ g of nuclear extract/well. A competition assay was done by premixing nuclear extracts for 30 min at 4°C with wild-type (WT) consensus and mutated consensus oligonucleotides provided in the kit before addition to the probe immobilized on the plate.

EMSA. The activator protein 1 (AP1) consensus oligonucleotide binding site for c-Jun homodimer and Jun/Fos heterodimeric complexes and AP1 mutant (AP1m) oligonucleotide (identical to AP1 consensus oligonucleotides with the exception of a CA to TG substitution in the AP1 binding motif) were obtained from Santa Cruz Biotechnology, Inc. The double-stranded oligonucleotides were labeled at the 5' end with [γ -³²P]ATP (Perkin Elmer) using T4 polynucleotide kinase (Gibco BRL). To prepare ³²P-labeled probe for RTA-AP1 and RTA-AP1m, single-strand oligonucleotides (66) were purchased from Invitrogen, annealed, and labeled at the 5' end with [γ -³²P]ATP (Perkin Elmer) using T4 polynucleotide kinase (Gibco BRL). Binding reactions were incubated on ice for 20 min and were performed in a 20- μ l reaction volume containing 50 mmol/liter NaCl, 10 mmol/liter Tris-HCl, pH 7.5, 1 mmol/liter MgCl₂, 0.5 mmol/liter EDTA, 0.5 mmol/liter dithiothreitol, 9% (vol/vol) glycerol, 1 μ g of poly(dI-dC), 6 μ g of nuclear extract, and labeled probe (10,000 cpm). The resulting DNA-protein complexes were then size fractionated from the free DNA probe by electrophoresis at 200 V on a 6% native polyacrylamide gel (acryl/bisacrylamide ratio of 29/1) containing 2.5% glycerol, 50 mmol/liter Tris-HCl, pH 8.5, 0.4 mol/liter glycine, and 2.7 mmol/liter EDTA. These gels were dried at 80°C for 30 min, exposed to Kodak X-ray film, and visualized with a PhosphorImager (Molecular Dynamics), and the band intensities were calculated. A competition electrophoretic mobility shift assay (EMSA) was performed by adding a 100 $\times$ molar excess of unlabeled double-stranded oligonucleotide probes, AP1 oligonucleotide (WT), and RTA-AP1 oligonucleotide, for respective treatments.

The nucleotide sequences of the annealed DNA probes used for AP1 consensus, AP1 mutated, RTA-AP1, and RTA-AP1m are shown in Fig. 1.

RESULTS

KSHV induces the p42/p44 ERK1/2 MAPK pathway in a dose-dependent manner. In our previous studies, we observed a rapid induction of the PI-3K-PKC ζ -MEK-ERK signal pathway in target cells infected with KSHV at an MOI of 5 (44). In this study, we examined the ability of KSHV at different MOIs (viral DNA copy/cell) to induce ERK1/2 phosphorylation. When serum-starved HFF cells infected for 30 min were analyzed with anti-phospho-ERK1/2 antibodies for ERK1/2 phosphorylation, compared to uninfected cells (Fig. 2A, top band,

AP1 Consensus Oligonucleotide

5' - CGC TTG ATG ACT CAG CCG GAA - 3'
3' - GCG AAC TAC TGA GTC GGC CTT - 5'

AP1 Mutant Oligonucleotide

5' - CGC TTG ATG ACT TGG CCG GAA - 3'
3' - GCG AAC TAC TGA ACC GGC CTT - 5'

RTA-AP1 Oligonucleotide

-87 -81

5' GATC CTA CCG GCG ACT CAT TAA GCC-74
-94 GAT GGC CGC TGA GTA ATT CGG CTAG

RTA-AP1m Oligonucleotide

5' GATC CTA CCG GCG GAT CCT TAA GCC
GAT GGC C GC CTA GGA ATT CGG CTAG

FIG. 1. Nucleotide sequences of the annealed DNA probes for AP1 consensus, AP1 mutants, RTA-AP1, and RTA-AP1m.

lane 1), a dose-dependent increase in ERK1/2 phosphorylation was observed in infected cells (Fig. 2A, top band, lanes 2 to 8). About 1.2- to 1.4-fold induction was observed at a low MOI, which increased to 2- to 5-fold at an MOI of 2 to 10. Equal loading of lysate was confirmed by Western blot reactions with antibodies against the total ERK2 protein (Fig. 2A, lower band). These results demonstrated the steady-state level of endogenous ERK2, thus suggesting that KSHV infection was activating the endogenous or preexisting ERK. The maximum activation by KSHV infection at an MOI of 5 probably reflects target cell saturation. Induction of ERK1/2 by a low MOI of KSHV suggested that this phosphorylation event is probably physiologically relevant.

To determine the kinetics of ERK1/2 by KSHV, lysates from serum-starved cells infected with KSHV at an MOI of 5 for different time points were analyzed (Fig. 2B, top band, lanes 2 to 6). Incubation of cells with 20% FBS for 15 min induced a rapid phosphorylation of ERK in 15 min (Fig. 2B, top band, lane 7). Similar to our earlier reports, compared to uninfected control cells (Fig. 2B, top band, lane 1), a 2.5- and 3-fold increase in ERK phosphorylation was observed at 5 and 15 min p.i., respectively (Fig. 2B, top band, lanes 2 and 3). This activation reached a maximum of fourfold by 30 min (Fig. 2B, top band, lane 4) and decreased to the basal level at 60 and 120 min postinfection (Fig. 2B, lanes 5 and 6). Equal loading of total lysate between the treatments was confirmed by Western blot reactions with antibodies against the total ERK2 protein (Fig. 2B, lower band).

KSHV induces the p42/p44 ERK1/2 MAPK in the presence of serum. To mimic the effect of KSHV during the initial stage of infection in quiescent cells and to determine the induction of preexisting signal pathways, we routinely use serum-starved confluent target cells (44, 54). To determine whether KSHV-induced ERK phosphorylation is a specific response to the infectious process or a compensatory mechanism induced by serum starvation, HFF cells in the presence of serum were

FIG. 2. Induction of ERK1/2 by KSHV. (A) ERK1/2 phosphorylation by different MOIs of KSHV. (Top band) Serum-starved HFF cells were either uninfected (lane 1) or infected with at different MOIs (DNA copies/cell) of KSHV for 30 min at 37°C (lanes 2 to 8). Ten micrograms of cell lysates was resolved by SDS-10% PAGE, subjected to Western blotting, and reacted with anti-phospho ERK1/2 antibodies. (B) Kinetics of ERK1/2 induction by KSHV. (Top band) Serum-starved HFF cells were either uninfected (lane 1) or infected at an MOI of 5 with KSHV for indicated time points (lanes 2 to 6), or treated with 20% FBS for 15 min (lane 7). Cell lysates were resolved by SDS-10% PAGE and subjected to Western blotting with anti-phospho ERK1/2 antibodies. (C) ERK1/2 phosphorylation in serum-fed HFF cells. (Top band) HFF cells grown in the presence of serum were either uninfected (lane 5) or infected with KSHV at an MOI of 5 for indicated time points (lanes 4 to 1). Cell lysates were resolved by SDS-10% PAGE and subjected to Western blotting with anti-phospho ERK1/2 antibodies. The bottom bands of panels A, B, and C show membranes that were stripped and reprobed with anti-ERK2 antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence reactions, and the band intensities were assessed. The ERK1/2 phosphorylation in the uninfected cells was considered 1 for comparison and expressed as an increase in phosphorylation (*n*-fold) of ERK1/2. Each point represents the average $\pm$ the standard deviation of three experiments. (D) KSHV infection triggers nuclear translocation of phosphorylated ERK1/2. Serum-starved HFF cells in eight-well chamber slides were either uninfected (frames 1 and 4) or infected with KSHV (MOI, 10) for 30 min (frames 2 and 5), or incubated with 20% FBS for 30 min (lanes 3 and 6), and then collected, permeabilized, and stained with anti-phospho p42/p44 MAPK monoclonal antibodies and anti-p42/p44 MAPK polyclonal antibodies recognizing phosphorylated (activated) and total p42/p44 MAPKs, respectively. Magnification, $\times 100$.

infected with KSHV at an MOI of 5. Compared to the uninfected serum-starved cells (Fig. 2B, lane 1), the background ERK1/2 activity in the uninfected cells with serum was much higher (Fig. 2C, top band, lane 5). Despite this background level, similar to serum-starved cells, KSHV induced a twofold increase in ERK phosphorylation as early as 5 min p.i. (Fig. 2C, top band, lane 4), which was maintained at 1.8-fold over background at 15, 30, and 60 min p.i. (Fig. 2C, top band, lanes 3, 2, and 1, respectively). Compared to serum-starved infected cells (Fig. 2A and B), levels of ERK phosphorylation did not decrease to the basal level at 60 min in serum-fed KSHV-infected cells. Equal loading of total lysate between the treatments and the steady-state level of endogenous ERK2 were demonstrated by Western blot reactions with antibodies against the total

ERK2 protein (Fig. 2C, lower band). These results confirmed the activation of endogenous or preexisting ERK by KSHV in the target cells and suggested that ERK induction is a consequence of infection and not a response to serum starvation.

KSHV triggers the rapid nuclear translocation of p42/p44 MAPK. Phosphorylation and dephosphorylation play significant roles in the signaling cascades, and the subcellular location of a phosphorylated protein is important for its activity and inactivity. The p44 and p42 MAPK isoforms (ERK1 and ERK2) are serine/threonine kinases, and their activity is positively regulated by phosphorylation mediated by MEK1 and MEK2, which phosphorylate ERK1T²⁰² and Y²⁰⁴ and ERK2 T¹⁸⁵ and Y¹⁸⁷ to activate their kinase activities (71). Among all the members of the MAPK signaling cascade, ERK1 and

FIG. 2—Continued.

ERK2 have been shown to be the only key mediators of signal transduction transmitting signals from the cell surface to the nucleus. Upon signal induction, the MEK remains cytoplasmic, whereas ERKs anchored to MEK in the cytoplasm of resting cells translocate to the nucleus, a process which is rapid, reversible, and controlled by the strict activation of the MAPK cascade (34, 63).

Since KSHV induced ERK1/2 early during infection, we examined the infected cells using monoclonal antibody against the MAP kinase synthetic diphosphopeptide. This antibody specifically recognized the active, doubly phosphorylated forms but not the inactive mono- and nonphosphorylated forms of ERKs (73). Infection with KSHV (MOI of 10) for 30 min induced the rapid nuclear translocation of phosphorylated ERK1/2 in >90% of infected cells; an example is shown in Fig. 2D, frame 2. This is similar to the translocation observed in cells treated with 20% FBS (Fig. 2D, frame 3). KSHV-induced ERK1/2 translocation correlated well with the rapid phosphorylation of ERK1/2, as demonstrated in Fig. 2B. In contrast, appreciable amounts of phosphorylated p42/p44 MAPKs were not detected in the nuclei of uninfected cells (Fig. 2D, frame 1). When the subcellular localization of total p42/p44 MAPKs was observed in these cells, the inactive p42/p44 MAPKs pool was primarily located in the cytoplasm (Fig. 2D, frames 4, 5, and 6). These results demonstrated that KSHV triggers the rapid nuclear entry of phosphorylated p42/p44 MAPKs in the infected cells.

UV-inactivated KSHV induces the p42/p44 ERK1/2 MAPK pathway. To determine whether KSHV gene expression is essential for ERK1/2 activation, replication-incompetent KSHV was prepared by UV irradiation. After 20 min of UV exposure, binding of [³H]thymidine-labeled KSHV to HFF cells was not affected (data not shown). When KSHV entry into the target cells was measured by quantitative real-time DNA PCR (32), UV-inactivated virus entered the target cells as efficiently as

the untreated virus (Fig. 3A). As demonstrated previously (32), immediately following infection with live KSHV (10 DNA copies/cell), a quantitative increase in expression of the latency-associated ORF 73 gene was seen, as well as expression of a limited number of the lytic cycle-associated ORF 50, K8, and K5 genes (Fig. 3B, left). In contrast, no such increase in KSHV gene expression was observed in cells infected with equal MOIs of UV-inactivated KSHV (Fig. 3B, right). However, similar to live KSHV (Fig. 3C, upper band, lane 3), a robust ERK1/2 activation was observed in target cells incubated with UV-inactivated KSHV, and about 2.3-, 3.7-, 4.8-, and 2.7-fold ERK1/2 activations were detected at 5, 15, 30, and 60 min p.i., respectively (Fig. 3C, upper band, lanes 4 to 7). These results suggested that the early kinetics of ERK activation of KSHV was not dependent on KSHV gene expression but probably depended upon signal induction mediated by KSHV envelope glycoprotein interactions with host cell receptor molecule(s) during the binding and entry stages of infection.

KSHV envelope glycoprotein gpK8.1A induces the maximal ERK activity. The major KSHV envelope glycoproteins gB and gpK8.1A bind to the cell surface heparan sulfate molecules (2, 3, 64), and gB interacts with the $\alpha\beta 1$ integrin molecule (4). Soluble gB but not soluble gpK8.1A induced the integrin-dependent preexisting FAK-Src-PI-3K-RhoGTPase signal pathways and induced the substrate adhesion of target cells (54, 64). To determine the role of gB and gpK8.1A in ERK1/2 activation, lysates from HFF cells induced for 30 min with different concentrations of Δ TMgB, Δ TMgB-RGA, Δ TMgpK8.1A, and ORF 73 proteins were analyzed for ERK1/2 phosphorylation (Fig. 4A). No induction was seen in cells incubated with DMEM, and incubation for 15 min with DMEM containing 20% FBS induced a rapid sixfold phosphorylation of ERK (Fig. 4A, top band, lanes 1 and 2). Phosphorylation of ERK1/2 was not observed in cells incubated with various concentrations of Δ TMgB, Δ TMgB-RGA, and ORF 73 proteins (Fig. 4A, top

FIG. 3. Effect of UV inactivation on ERK1/2 induction. (A) Entry of UV-inactivated KSHV. HFF cells were infected with UV-inactivated KSHV or live KSHV (10 DNA copies/cell). After a 2-h incubation, cells were washed twice with PBS to remove the unbound virus, treated with trypsin-EDTA for 5 min at 37°C to remove the bound but noninternalized virus, and washed, and total DNA was isolated. This was normalized, and internalized KSHV genomes per 100 ng of DNA samples, as measured by ORF 73 copies, were estimated by real-time DNA PCR. The C_T values were used to plot the standard graph and to calculate the relative copy numbers of viral DNA in the samples. Data are presented as percentages of internalized viral DNA. Each reaction was done in duplicate, and each point represents the average $\pm$ standard deviation of three experiments. (B) Effect of UV inactivation on the kinetics of KSHV gene expression. HFF cells were infected with UV-inactivated KSHV or KSHV (10 DNA copies/cell). At indicated time points p.i., RNA was isolated and treated with DNase I, and 250 ng of DNase-treated RNA was subjected to real-time RT-PCR with ORF 73, ORF 50, K5, and K8 gene-specific primers and TaqMan probes (32). The relative copy numbers of viral transcripts were calculated using a standard graph generated by using known concentrations of DNase-treated in vitro transcribed ORF 73, ORF 50, K5, and K8 transcripts in real-time RT-PCR and normalized with GAPDH. Each reaction was done in duplicate and each point represents the average $\pm$ standard deviation of three independent experiments. (C) Kinetics of ERK1/2 induction by UV-inactivated KSHV. (Top band) Serum-starved HFF cells were either uninfected (lane 1) or infected with KSHV at an MOI of 10 (lane 3) or with UV-inactivated KSHV for indicated time points (lanes 4 to 7), or treated with 20% FBS for 15 min (lane 2). Membranes were stripped and reprobed with anti-ERK2 antibodies (bottom band). P-ERK, phosphorylated ERK.

band, lanes 3 to 6, 11 to 14, and 15, respectively). In contrast, Δ TMgpK8.1A induced ERK phosphorylation in a dose-dependent manner (Fig. 4A, top band, lanes 7 to 10), and maximum induction was observed with 2 μ g/ml of Δ TMgpK8.1A (Fig. 4A, lane 9). Incubation with even low (0.5 μ g/ml) concentrations of Δ TMgpK8.1A could induce a twofold ERK phosphorylation. These results demonstrated that like KSHV infection, gpK8.1A was activating the endogenous or preexisting ERK.

Kinetics of ERK1/2 induction by Δ TMgpK8.1A. Lysates from HFF cells induced with 2 μ g/ml of Δ TMgpK8.1A for different time points were analyzed for ERK activity in an in vitro immune-complex kinase assay. Quantitation of 32 P-MBP (Fig. 4B, top band) as a measure of ERK activity showed that compared to uninduced cells (Fig. 4B, lane 1, and 4C),

Δ TMgpK8.1A induced a 2.5- and 3-fold increase in ERK phosphorylation at 5 and 15 min of incubation, respectively (Fig. 4B, lanes 2 and 3, and C). This activation reached a maximum of fivefold by 30 min (Fig. 4B, lane 4, and C) and decreased close to the basal levels by 60 to 120 min (Fig. 4A, lanes 5 to 6, and C). FBS used as a positive control induced a rapid activation of ERK in 15 min (Fig. 4A, top panel, lane 7, and C). In contrast, incubation with either Δ TMgB (30 min), or with Δ TMgB-RGA (30 min) had no effect on ERK activity (Fig. 4A, top band, lanes 8 and 9, respectively). Western blot analysis of the same lysates with anti-phospho ERK1/2 antibodies demonstrated the increase in the phosphorylated forms of ERK1/2 with kinetics similar to the kinase assay (Fig. 4B, middle band), thus confirming the induction of ERK activity. Equal loading

FIG. 4. Induction of MAPK-ERK1/2 by KSHV gpK8.1A and gB. (A, top band) Serum-starved HFF cells were either uninduced (lane 1) or induced with 20% FBS for 15 min (lane 2) or treated with different concentrations of ΔTMgB (lanes 3 to 6), ΔTMgPK8.1A (lanes 7 to 10), or ΔTMgB-RGA (lanes 11 to 14), or 4.0 μg/ml of ORF 73 (lane 15) proteins for 30 min. The increase in phosphorylation (*n*-fold) was quantitated as described in the legend Fig. 2A and C. Bottom band, total ERK2 (B) Kinetics of ERK1/2 induction by ΔTMgPK8.1A. (Top band) Serum-starved cells were uninduced (lane 1) or induced with 2.0 μg/ml of ΔTM gpK8.1A for 5, 15, 30, 60, and 120 min (lanes 2 to 6, respectively), or treated with FBS for 15 min (lane 7), or treated with 2.0 μg/ml ΔTMgB for 30 min (lane 8), or treated with 2.0 μg/ml ΔTMgB-RGA for 30 min (lane 9). Equal protein concentrations of cell lysates were immunoprecipitated with anti-ERK2 antibodies, and immune complexes were incubated with [γ-³²P]ATP and myelin basic protein for 20 min at 30°C, boiled in sample buffer, and analyzed by SDS-12% PAGE. A portion of these immunoprecipitated samples were reacted in Western blot reactions with anti-total ERK2 antibodies (bottom band). Equal protein concentrations of cell lysates were reacted in Western blot reactions with anti-phospho ERK1/2 antibodies (middle panel). (C) Quantitation of ERK activity induced by ΔTMgPK8.1A. ³²P-MBP bands were scanned and the increase in phosphorylation (*n*-fold) was quantitated as described in the legends of Fig. 2A and C. Each point represents the average ± the standard deviation of three experiments. p-ERK1/2, phosphorylated ERK1/2.

of total lysate between different treatments was confirmed by Western blot reactions with antibodies against the total ERK1/2 protein (Fig. 4B, bottom band).

Cell type specificity of ERK1/2 activation. To determine whether the ERK activation is a cell type-specific phenomenon, ERK1/2 phosphorylation was tested in HMVEC-d (Fig.

5A). Compared to HFF cells, the endogenous level of phospho-ERK1/2 in HMVEC-d was much lower (Fig. 5A, top band, lane 14). Similar to HFF cells and in comparison to uninduced cells (Fig. 5A, lane 14), treatment of serum-starved HMVEC-d with 2 μg/ml of ΔTMgPK8.1A for 5 and 15 min resulted in about 1.5- and 5-fold increases in ERK phosphorylation (Fig.

FIG. 5. Characterization of ERK1/2 induction. (A) Kinetics of ERK1/2 induction in HMVEC-d. Serum-starved HMVEC-d were either uninduced (lane 14) or induced with DMEM containing 20% FBS for 15 min (lane 13) or treated with 2.0 μ g/ml of Δ TMgpK8.1A (lanes 12 to 10), 2.0 μ g/ml of Δ TMgB (lanes 9 to 7), Δ TMgB-RGA (lanes 6 to 4), or 2.0 μ g/ml of Δ TM gpK8.1A (lanes 3 to 1) for indicated time points. Equal protein concentrations of cell lysates were resolved by SDS-PAGE and subjected to Western blotting with anti-phospho ERK1/2 antibodies (top band). Membranes were stripped and reprobed with anti-ERK2 antibodies (bottom band). The increase in phosphorylation (n -fold) was quantitated as described in the legends of Fig. 2A and C. Each blot is representative of at least three independent experiments. (B) Inhibition of ERK induction by heparin. Serum-starved HFF were either uninduced (lane 6) or induced with Δ TMgpK8.1A preincubated with 1, 10, and 100 μ g/ml of heparin (lanes 4 to 2, respectively) or 100 μ g/ml of chondroitin sulfate C (lane 1). Cell lysates were reacted in Western blots with anti-phospho ERK1/2 antibodies. Membranes were stripped and reprobed with anti-ERK2 antibodies (bottom band). ERK activity in cells incubated with Δ TMgpK8.1A protein alone was considered 100%, and data are presented as the percentage of inhibition of ERK phosphorylation. (C) Inhibition of ERK induction by Δ TMgpK8.1A neutralizing antibodies. Serum-starved HFF were induced with 2 μ g/ml of Δ TMgpK8.1A for 30 min (lane 1), with gpK8.1A preincubated with different concentrations of anti-gpK8.1A MAb 4D6 (lanes 2 to 6), or with 100 μ g/ml of anti-KSHV ORF 59 MAb 11D1 (lane 7) before being added to the cells. Lysates were subjected to a kinase assay (top band) as described in the legend of Fig. 4B or immunoprecipitates were Western blotted with anti-phospho ERK1/2 antibodies (bottom band). Equal protein concentrations of cell lysates were probed with anti-total ERK2 antibodies (bottom band). (D) Quantitation of ERK activity induced by Δ TMgpK8.1A. ³²P-MBP bands were scanned and quantitated. ERK activity in HFF cells incubated with 2 μ g/ml of Δ TMgpK8.1A protein was considered 100%, and data are presented as percentage of inhibition of ERK phosphorylation. Each point represents the average $\pm$ the standard deviation of three experiments. p-ERK1/2, phosphorylated ERK1/2.

5A, lanes 12 and 11, respectively). However, unlike HFFs, the maximum induction of ERK1/2 was observed at 15 min in HMVEC-d (Fig. 5A, lane 11), which decreased to twofold by about 30 min (Fig. 5A, lane 10). In contrast, incubation with either 2 μ g/ml of Δ TMgB-RGA or 2 μ g/ml ORF 73 for 5, 15, and 30 min had no effect on ERK phosphorylation in HMVEC-d (Fig. 5A, top band, lanes 6 to 1, respectively). Even though no ERK1/2 induction was observed in HFF cells induced with Δ TMgB, incubation of HMVEC-d for 15 min with Δ TMgB induced a twofold ERK1/2 phosphorylation (Fig. 5A, top band, lane 8). These results suggested that interaction of KSHV glycoproteins with cell surface receptors and the onset of signaling cascades differ from one cell type to another and

that KSHV gpK8.1A is the major inducer of ERK1/2 phosphorylation in HMVEC-d and HFF cells.

Specificity of ERK1/2 activity induced by Δ TMgpK8.1A. To confirm that the ERK1/2 phosphorylation was induced by purified soluble Δ TMgpK8.1A and not by contaminating protein(s), Δ TMgpK8.1A was preincubated with increasing concentrations of heparin or chondroitin sulfate for 90 min at 4°C before incubation with HFF cells. Incubation of Δ TMgpK8.1A with 1, 10, and 100 μ g/ml of heparin reduced the ERK1/2 induction in dose-dependent manner by 45%, 70%, and 79%, respectively (Fig. 5B, top band, lanes 4 to 2, respectively). Only 16% inhibition of ERK phosphorylation was seen with 100 μ g/ml of chondroitin sulfate (Fig. 5B, lane 1). The total ERK-2

levels remained same during different treatments (Fig. 5B, bottom band).

To confirm that we were detecting ERK1/2 induction driven by Δ TMgpK8.1A, Δ TMgpK8.1A was preincubated with anti-gpK8.1A MAb 4D6 antibodies or with anti-ORF 59 MAb 11D1 for 1 h at 37°C before incubation with HFFs. Incubation with anti-gpK8.1A antibodies reduced the ERK1/2 induction at 30 min in a dose-dependent manner (Fig. 5C, top band, lanes 2 to 6). Quantitation demonstrated a 21%, 33%, 64%, and 82% reduction in ERK1/2 induction at 40, 60, 80, and 100 μ g/ml concentrations, respectively (Fig. 5D). No inhibition was seen with anti-ORF 59 MAb even at a concentration of 100 μ g/ml (Fig. 5D and C, lane 7). Western blot analyses of immune complexes detected equal amounts of total ERK1/2 protein, thus demonstrating that equal ERKs were immunoprecipitated (Fig. 5C, bottom band). Preincubation of KSHV with 100 μ g/ml of anti-gB and -gpK8.1A antibodies reduced the ERK1/2 induction 59% and 67%, respectively (data not shown). These results demonstrated that ERK was specifically induced by soluble gpK8.1A or by KSHV infection and not by the contaminating host cell factors and/or LPS or any component from the medium.

MEK1/2 is an upstream inducer of Δ TMgpK8.1A-activated ERK1/2. Since MEK1/2 is an upstream kinase that induces ERK1/2, we used the phosphorylation-specific antibodies to examine MEK1/2 induction by Δ TMgpK8.1A. Compared to the uninduced HFF cells (Fig. 6A, top band, lane 2), phosphorylation of MEK1/2 by Δ TMgpK8.1A increased in a time-dependent manner, from twofold at 5 min to a peak activation of fivefold at 30 min, decreasing at 60 min (Fig. 6A, top band, lanes 3 to 6) and to background level at 90 min (data not shown). KSHV infection for 30 min also induced the MEK1/2 phosphorylation by sixfold (Fig. 6A, top band, lane 1). In contrast, the total MEK or β -actin levels remained unaffected (Fig. 6A, middle and bottom bands, respectively). The observed kinetics of MEK1/2 induction was similar to the kinetics of ERK1/2 induction (Fig. 4B and 6A).

To determine whether MEK1/2 is an upstream stimulator of ERK-MAPK activated during Δ TMgpK8.1A treatment, HFF cells were preincubated with different concentrations of U0126 for 1 h at 37°C and then incubated with Δ TMgpK8.1A (2 μ g/ml) for 30 min. U0126 is a potent and specific covalent binding inhibitor of MEK1/2, and shows little, if any, effect on the kinase activities of PKC, Abl, cRaf-1, MEKK, ERK, JNK, MKK-3, MKK-4/SEK, MKK-6, Cdk2, or Cdk4 (17). U0126 pretreatment inhibited the Δ TMgpK8.1A-induced ERK phosphorylation in a dose-dependent manner without affecting the total ERK levels (Fig. 6B, bottom band, lanes 2 to 5). Control cells treated either with dimethyl sulfoxide (data not shown) or U0124, an analogue of U0126 with no inhibitory effect on MEK1/2, failed to block ERK1/2 phosphorylation (Fig. 6B, top band, lane 1). These results demonstrated that ERK1/2 activation by Δ TMgpK8.1A is dependent upon MEK1/2 kinase. This is similar to our earlier results where we have demonstrated that MEK1/2 is an upstream inducer of KSHV-stimulated ERK1/2 pathway (44).

KSHV and Δ TMgpK8.1A activate ERK1/2 but not ERK5 and p38-MAPK. To determine whether gpK8.1A induces other MAPK pathways, phosphorylation-specific antibodies were used to examine the induction of big MAPK (ERK5) and LPS-

or stress-activated p38-MAPK. Unlike the other MAPK cascades that seem to serve one set of extracellular stimuli such as stress or mitogens, the ERK5 cascade is activated by both stress responses and mitogens (1, 29). The ERK5 cascade mediates its function mainly through the regulation of transcription by directly phosphorylating and activating several transcription factors including c-Myc (18), MEF2 family members (30), and c-Fos (28). Treatment of HFF cells with 0.5 M sorbitol used as positive control induced the phosphorylation of ERK5 (Fig. 6C, top band, lanes 2 to 5), and the total level of ERK5 was maintained constant in these cell lysates (Fig. 6C, bottom band, lanes 1 to 7). In contrast, no phosphorylation of ERK5 was seen in cells infected with KSHV or treated with Δ TMgpK8.1A (Fig. 6C, top band, lanes 6 and 7). Similarly, phosphorylation of p38-MAPK was not observed with KSHV infection or with Δ TMgpK8.1A (Fig. 6D, bottom band, lanes 6 and 7). Treatment with 0.5 M sorbitol induced an appreciable level of p38-MAPK phosphorylation (Fig. 6D, top band, lanes 2 to 4). In contrast, the total p38 or β -actin levels remained unaffected (Fig. 6D, middle and bottom bands, respectively). These results suggested that KSHV and Δ TMgpK8.1A preferentially induced the MEK1/2-ERK1/2 pathway.

KSHV-induced MEK1/2 and ERK1/2 do not play a role in the internalization of virus. In our earlier studies, in cells preincubated with U0126 and ERK-antisense oligonucleotides, we observed the neutralization of GFP-KSHV infectivity as measured by the immunofluorescence assay measurement of GFP expression (44). Since this inhibition was observed without affecting KSHV binding to the target cells (44), to determine whether this is due to a role of ERK1/2 in viral internalization, we carried out a quantitative real-time DNA PCR assay (32). By this method, we have previously shown that internalized viral DNA could be detected in HFF cells as early as 5 min p.i., increasing rapidly during the first 60 to 90 min of infection and reaching a plateau at around 90 to 120 min p.i. (32). Preincubation of KSHV with 100 μ g of heparin per ml blocked the viral DNA entry by more than 90% (Fig. 7A). Treatment of HFF cells with the U0126 did not show any significant reduction of KSHV DNA internalization (Fig. 7A). In contrast, preincubation of cells with the PI-3K inhibitor LY294002 at 50 and 100 μ M concentrations reduced the internalization by about 64 to 79% (Fig. 7A), which reaffirmed our earlier conclusion that PI-3K plays a role in the entry stage of KSHV infection (44, 54). These results, together with inhibition of infectivity by the inhibitors of MEK (44, 54), suggest a role for activation of the MEK pathway subsequent to the virus internalization stage of infection.

KSHV-induced MEK1/2 and ERK1/2 do not play a significant role in the nuclear delivery of viral DNA. HFF cells were preincubated with 10 μ M U0126 or U0124, 50 μ M LY294002, or 300 ng/ml of CdTxA at 37°C for 1 h and then were infected with KSHV at 37°C in the presence of inhibitor. Infected cell nuclei were isolated and checked for purity, and viral DNA copy numbers were quantified by real-time PCR (42). As shown before (42), treatment with CdTxA (specifically affecting Rho-GTPases) and LY294002 significantly blocked viral DNA delivery to the nucleus by about 64% and 71%, respectively (Fig. 7B). Compared to LY294002 and CdTxA, no significant inhibition by ERK inhibitor U0126 and by its structural analogue U0124 was observed (Fig. 7B). The moderate inhi-

FIG. 6. Δ TMgpK8.1A induces MEK1/2. (A) Kinetics of MEK1/2 induction. (Top band) Serum-starved HFF were either infected with KSHV (5 DNA copies/cell) for 30 min (lane 1), uninduced for 60 min (lane 2), induced with Δ TMgpK8.1A for 5, 15, 30, and 60 min (lanes 3 to 6, respectively), or treated with DMEM containing 20% FBS for 15 min (lane 7). Western-blotted equal protein concentrations of cell lysates were reacted with anti-phospho MEK1/2 antibodies. Membranes were stripped and reprobed with anti-MEK1/2 antibodies (middle band) or with anti- β -actin antibodies (bottom band). (B) Inhibition of MEK1/2 inhibits the ERK1/2 activity induced by Δ TMgpK8.1A. Serum-starved HFF cells were uninduced (lane 7) or induced with Δ TMgpK8.1A for 30 min (lane 6). Cells were also preincubated with either MEK1/2 inhibitor U0126 (lanes 2 to 5) or MEK1/2 inhibitor analogue U0124 (lane 1) for 1 h at 37°C and then induced with Δ TMgpK8.1A for 30 min in the presence of inhibitors. Cell lysates were subjected to Western blotting and reacted with anti-phospho ERK1/2 antibodies (top band) or with anti-ERK2 antibodies (bottom band). ERK activity in cells incubated with Δ TMgpK8.1A protein alone was considered 100%, and data are presented as percentage of inhibition of ERK phosphorylation. (C) KSHV and Δ TMgpK8.1A activate ERK1/2 but not ERK5. (Top band) Serum-starved HFF cells were either uninduced (lane 1), induced with 0.5 M sorbitol for different time points (lanes 2 to 5), or infected with KSHV (5 DNA copies/cell) for 30 min (lane 6) or 2 μ g/ml of Δ TMgpK8.1A for 30 min (lane 7). Cell lysates were subjected to Western blotting and reacted with anti-phospho-ERK5 antibodies. Membranes were stripped and reprobed with anti-total ERK5 antibodies (bottom band). Each blot is representative of at least three independent experiments. The ERK5 phosphorylation in the uninduced cells was considered 1 for comparison. (D) KSHV and Δ TMgpK8.1A activate ERK1/2 but not p38 MAPK. (Top band) Serum-starved HFF were either uninduced (lane 1), induced with 0.5 M sorbitol for the indicated times (lanes 2 to 5), infected with KSHV (5 DNA copies/cell) for 30 min (lane 6), or treated with 2 μ g/ml of Δ TMgpK8.1A for 30 min (lane 7). Cell lysates were subjected to Western blotting and reacted with anti-phospho p38 antibodies. Membranes were stripped and reprobed with anti- β -actin antibodies (bottom band). Each blot is representative of at least three independent experiments. The p38 phosphorylation in the uninduced cells was considered 1 for comparison.

bition of nuclear delivery of KSHV DNA observed with U0126 could be due to the role of ERK1/2 in myosin contraction and actin polymerization (20, 58). Taken together, these results demonstrated that ERK does not play a major role in the nuclear delivery of viral DNA and suggested that our previous observation of inhibition of KSHV infectivity by ERK inhibitor (44) could be predominantly due to ERK's role in viral gene expression.

Inhibition of ERK1/2 blocks the expression of KSHV latent ORF 73 gene expression. To analyze the potential role of ERK1/2 activation in KSHV gene expression, HFF cells and HMVEC-d were preincubated with U0126 at 37°C for 1 h and infected with KSHV for 2 h, and viral messages collected at different times p.i. were quantitated by real-time RT-PCR. We have previously shown that immediately after infection of HMVEC-d and HFF cells, KSHV expressed the latent ORF 73

FIG. 7. Effect of ERK1/2 inhibition on virus entry and nuclear delivery of viral DNA. (A) Inhibition of ERK1/2 does not block viral DNA internalization. HFF cells or HFF cells incubated with different concentrations of inhibitors for 1 h at 37°C were infected with KSHV at 10 DNA copies/cell. For a control, virus was preincubated with 100 µg/ml of heparin for 1 h at 37°C before being added to the cells. After a 2-h incubation, cells were washed twice with PBS to remove the unbound virus, treated with trypsin-EDTA for 5 min at 37°C to remove the bound but noninternalized virus, and washed, and total DNA was isolated. This was normalized, and number of KSHV genome copies were estimated by real-time DNA PCR of ORF73. The C_T values were used to plot the standard graph and to calculate the relative copy numbers of viral DNA in the samples. Data are presented as the percentage of inhibition of KSHV DNA internalization obtained when the cells were incubated with virus alone. Each reaction was done in duplicate, and each bar represents the mean $\pm$ standard deviation of three experiments. **, statistically significant ($P < 0.001$). (B) Inhibition of MEK1/2 and ERK 1/2 does not influence the nuclear delivery of KSHV DNA. HFF cells or HFF cells preincubated for 1 h with nontoxic doses of LY294002 (50 µM), CdTx-A (300 ng/ml), U0126 (10 µM), or U0124 (10 µM) were infected with 5 DNA copies/cell of KSHV for 3 h in the presence of inhibitors. Nuclear fractions were purified and assessed for purity, and total DNA was normalized to contain 100 ng/5 µl was analyzed by real-time DNA PCR using KSHV ORF73 primers. Copy standards and nontemplate controls were run in parallel. A standard graph generated by real-time PCR of known concentrations of a cloned ORF 73 gene was used to calculate the relative viral DNA copy numbers. Data are presented as percentages inhibition of KSHV DNA associated with the infected cell nuclei relative to cells incubated with virus alone. Each reaction was done in duplicate, and each bar represents the mean $\pm$ standard deviation of three experiments. **, statistically significant ($P < 0.001$).

and 72 and K13 genes as well as the lytic cycle switch ORF 50 gene (32). In this present study also we observed a steady increase in ORF 73 gene expression in HMVEC-d and HFF cells (Fig. 8A and B). This expression was significantly reduced in U0126-pretreated HMVEC-d at 1, 2, 4, and 8 h p.i. with about 83%, 87%, 94%, and 95% reduction, respectively (Fig. 8C). Similarly, ORF 73 expression was significantly reduced in U0126-pretreated HFF cells at 2, 4, and 8 h p.i. with about 81%, 86%, and 93% reduction, respectively (Fig. 8D). The effect of ERK inhibitor on ORF 73 gene expression was maintained throughout the observed period with about 77% reduction even at 24 h p.i. (data not shown). The specificity of these reactions was demonstrated by the absence of contaminating DNA in any of the DNase-treated RNA samples. Even though we observed slight differences in the copy numbers of transcripts in different experiments and batches of viruses, the patterns of gene expression were closely similar and highly reproducible.

As a control for these experiments, we also analyzed the effect of the ERK inhibitor U0126 on the expression of ORF 73 in BCBL-1 cells with an established KSHV latent infection. GFP-KSHV-BCBL-1 cells were serum starved for 8 h and then left untreated or treated with U0126 for 1 h, 2 h, and 3 h. The expression of ORF 73 and GFP transcripts was measured by real-time RT-PCR and normalized with GAPDH transcripts. Similar to de novo infection, the copy numbers of ORF 73 transcript were inhibited by 16.5%, 27%, and 34.5% at 1, 2, and 3 h of pretreatment with 10 µM U0126, respectively (data not shown). Similarly, GFP expression was also inhibited by 29%, 38%, and 60% at 1, 2, and 3 h of pretreatment with 10 µM U0126, respectively (data not shown). These results suggested an active role of the ERK1/2 pathway in transcription initiation of KSHV latency transcripts.

Inhibition of ERK1/2 blocks the expression of KSHV lytic ORF 50 gene expression. The 110-kDa KSHV immediate early ORF 50 (RTA) protein is a transcriptional activator and acts as a molecular switch for KSHV reactivation (17, 21, 40, 53, 59, 68). As demonstrated previously (32), we observed a steady increase in ORF 50 gene expression in HMVEC-d and HFF cells with slightly varying kinetics (Fig. 8A and B). Copy numbers of ORF 50 transcripts reached a peak at 2 h p.i. in HMVEC-d and declined rapidly at subsequent time points (Fig. 8A). In contrast, in HFF cells, the peak level of ORF 50 gene expression was observed at 8 h p.i., followed by a sharp decline by 24 h p.i. (Fig. 8B). The observed ORF 50 expression was significantly reduced in U0126-pretreated cells at all time points (Fig. 8C and D). About 20%, 84%, 80%, and 60% reduction was observed at 1, 2, 4, and 8 h p.i. in HMVEC-d, respectively (Fig. 8C). In HFF cells, about 66%, 60%, and 58% reduction was observed at 2, 4, and 8 h p.i., respectively (Fig. 8D). After 24 h p.i., about 40% inhibition in ORF 50 expression was observed in HFF cells pretreated with MEK inhibitor (data not shown). These results suggested that the ERK1/2 pathway also plays a role in the transcription initiation of KSHV ORF 50 transcripts.

Inhibition of ERK1/2 blocks the expression of KSHV immediate early lytic K8 and v-IRF2 genes but not K5 gene expression. Our studies have shown that in addition to the latent and lytic ORF 50 genes, KSHV also expressed a limited number of lytic cycle genes early during infection (32). Though the detec-

FIG. 8. Kinetics of KSHV ORF 73 and ORF 50 gene expression in the presence of U0126. (A and B) HMVEC-d and HFF cells were infected with KSHV (10 DNA copies/cell) for 2, 4, 8, and 24 h; RNA was isolated and treated with DNase I for 1 h. A total of 250 ng of DNase-treated RNA was subjected to real-time RT-PCR with ORF 73 and 50 gene-specific primers and TaqMan probes (32). Standard graphs generated using known concentrations of DNase-treated in vitro transcribed ORF 73 and ORF 50 transcripts were used to calculate the relative copy numbers of viral transcripts and were normalized with GAPDH. Each reaction was done in duplicate, and each point represents the average $\pm$ standard deviation of three independent experiments. **, statistically significant ($P < 0.001$). (C and D) Histogram depicting the percent inhibition of KSHV ORFs 73 and 50 expression in the presence of U0126. Data represent the average $\pm$ standard deviation of three experiments. **, statistically significant ($P < 0.001$).

tion of viral transcripts by gene array in the earlier study could also represent messages carried in the virion particles, further examination revealed a steady quantitative increase in early lytic K5, K8, and v-IRF2 gene expression in the infected HMVEC-d and HFF cells with various kinetics (Fig. 9A and B). KSHV ORF 50 gene activates the expression of K8 and v-IRF2 genes (35, 66, 67, 59), and, as expected, the expression kinetics of K8 and v-IRF2 genes (Fig. 9A and B) followed the ORF 50 expression kinetics (Fig. 8A and B). Expression of K8 and v-IRF2 reached a peak around 2 h and 8 h p.i. in HMVEC-d and HFF cells, respectively, and declined sharply thereafter (Fig. 9A and B). Pretreatment of HMVEC-d with U0126 inhibited the expression of K8 by about 37%, 73%, 44% and 7% at 1, 2, 4, and 8 h p.i., respectively (Fig. 9C), and by about 83%, 97%, 71%, and 33% at 2, 4, 8, and 24 h, respectively, in HFF cells (Fig. 9D). U0126 pretreatment also affected the v-IRF2 expression by about 32%, 68%, 59%, and 12% at 1, 2, 4, and 8 h p.i., respectively, in HMVEC-d (Fig. 9C) and by about

50%, 45%, 95%, and 24% at 2, 4, 8, and 24 h p.i., respectively, in HFF cells (Fig. 9D). Maximum inhibition correlated with the time point at which maximum expression of K8 and v-IRF2 genes was observed. Unlike K8 and v-IRF2, ERK1/2 inhibition had minimal impact on K5 gene expression in HMVEC-d and HFF cells (Fig. 9C and D). K5 was inhibited by about 23%, 16%, 5%, and 4% at 1, 2, 4, and 8 h, respectively, in HMVEC-d (Fig. 9C) and by about 12% and 37% at 4 h and 8 h, respectively, in HFF cells (Fig. 9D). Since expression of K8 and v-IRF2 depends upon the expression of ORF 50 (35, 59, 66, 67), these results suggested that the ERK1/2 pathway plays a pivotal role in KSHV transcription initiation. The limited impact by ERK1/2 inhibition over K5 gene expression may probably be due to the ORF 50-dependent and -independent expression of the K5 gene (46). These differential effects over KSHV gene expression by U0126 also suggested the specificity of the observed reduction in viral RNA copy numbers.

FIG. 9. Kinetics of KSHV K5, K8, and v-IRF-2 in the presence of ERK inhibitor U0126. (A and B) DNase-treated total RNAs isolated from HMVEC-d and HFF cells infected with KSHV (10 DNA copies/cell) with and without U0126 were subjected to real-time RT-PCR as described in the legend of Fig. 8. (C and D) Histogram depicting the percent inhibition of KSHV ORF expression in the presence of U0126. Data represent the average $\pm$ standard deviation of three experiments.

MEK1/2-ERK1/2 inhibition blocks the expression of some of the host genes induced by KSHV infection. Our recent studies have demonstrated that early during infection of HMVEC-d and HFF cells (2 and 4 h p.i.), KSHV reprogrammed the transcriptional machinery regulating a variety of cellular processes. To determine the effect of ERK1/2 inhibition over KSHV-induced host cell gene induction, we used semiquantitative RT-PCR analysis to analyze a limited number of KSHV-induced genes such as vascular endothelial growth factor (VEGF), heparin binding epidermal growth factor (HB-EGF), IE-3, MAP3K8, intercellular adhesion molecule 1 (ICAM-1), and dual specificity phosphatase 5 (DUSP5), since ERK is known to have a direct or indirect role in the induction of these genes (43). As an example, RT-PCRs for DUSP5 gene transcripts are shown in Fig. 10A).

KSHV infection induced the expression of the DUSP5 gene in the infected HMVEC-d by about 10-fold at 2 and 4 h p.i. and by about 10- and 16-fold in HFF cells at 2 and 4 h, respectively (43). Similarly, the RT-PCR product from infected-cell RNA was readily detectable by 20 cycles of 2-h samples, whereas it was detectable only by 26 cycles in U0126-treated infected HFF cells (Fig. 10A, rows 1 and 2). DUSP5 RT-PCR product

was readily detectable by 23 cycles of 4-h samples from KSHV-infected as well as U0126-pretreated HFF cells (Fig. 10A, rows 3 and 4). In contrast, the amplification of the constitutively expressed β -actin gene reached the linear phase equally in both KSHV-infected and U0126-treated samples starting from a sample obtained at cycle 17 (Fig. 10B). Quantitation of RT-PCRs (Fig. 10B) demonstrates the increase in expression (*n*-fold) of DUSP5 in KSHV-infected and U0126-treated HFF cells at 2 h. Similar semiquantitative RT-PCR time kinetics for VEGF, HB-EGF, IE-3, MAP3K8, and ICAM-1 genes were carried out (Fig. 10C). Analyses revealed that the expression of DUSP5 was inhibited by 60% at 2 h p.i. and only by about 20% at 4 h p.i.. Similarly, in the presence of U0126, expression of VEGF was inhibited by about 20% and 44% at 2 and 4 h p.i., respectively (Fig. 10C), compared to about 12- and 50-fold induction at 2 and 4 h p.i. in untreated KSHV-infected cells.

Pretreatment of HFF cells with U0126 had no effect on the expression of the MAP3K8 gene, which was shown to be induced by KSHV about threefold at about 4 h p.i. (43). Similarly, the IE-3 gene, whose expression was induced by KSHV by about seven- and eightfold at 2 and 4 h p.i., was not affected (Fig. 10C). The expression of HB-EGF was highest at 2 h p.i.

FIG. 10. Effect of ERK inhibitor U0126 on host gene expression. (A) Semiquantitative RT-PCR confirmation of DUSP5 gene expression in U0126-treated and untreated KSHV-infected HFF cells. DNase-treated total RNAs were subjected to RT-PCR using specific primers. Successive samples were removed from every three cycles (14 to 41) and resolved on agarose gel, and the change in DUSP5 expression (n -fold) was calculated after normalizing to the β -actin gene. Ethidium bromide-stained RT-PCR amplified DUSP5 gene products after gel electrophoresis are shown. Representative amplification of the β -actin gene and reactions of DNA-PCR (-RT) are shown. (B) Quantitation of differential amplification of KSHV-infected and U0126-treated HFF cell RNA samples. IDVs corresponding to the sum of pixel intensities after background corrections were recorded for both the KSHV-infected and U0126-pretreated samples at linear points on the amplification curve and changes expression were created after normalizing to the β -actin gene. Representative graph depicts the change in DUSP5 expression (n -fold) as calculated from the change in the IDV. (C) Histogram representing the percent inhibition of host genes induced by KSHV infection in the presence of U0126. Expression of the specific gene by KSHV at indicated time points was considered 100%.

(18-fold), and U0126 treatment did not affect this expression. However, when the induction was about fourfold at 4 h p.i., about 25% inhibition was seen with U0126. In contrast, about 17- and 14-fold induction of ICAM-1 expression was observed at 2 and 4 h p.i.; about 77% inhibition was observed after 2 h in U0126-pretreated HFF cells (Fig. 10C). These results suggested that activation of ERK is not only involved in the maintenance of viral gene expression but also in the activation of some of the host genes induced by KSHV early during infection. These differential effects over host cell gene expression by U0126 also suggested that the observed reductions in viral RNA copy numbers were not due to a nonspecific shutdown of host transcriptional apparatus.

KSHV-induced ERK plays a role in the activation of MAPK family transcription factors. Since our studies suggested that the ERK1/2 pathway plays a role in the transcription initiation of KSHV ORF 50 and ORF 73 transcripts and some cellular genes, to determine whether this is due to the ability of ERK1/2 to modulate a variety of host transcription factors, we

next examined whether KSHV infection induces the activity of various transcription factors. Using an ELISA-based technique, nuclear extracts from the uninfected and infected HFF cells were assessed for the ability of host transcription factors to bind to their respective wild-type DNA sequences. Infection of HFF cells with KSHV increased the activity of MAPK-regulated transcription factors, which was dependent upon the time of infection; the data in Fig. 11A show a representative example of the activity of MAPK-regulated c-Jun, STAT1 α , MEF2, c-Myc, ATF-2, and c-Fos in HFF cells infected with KSHV for 120 min. The levels of these transcription factors were barely detectable in the serum-starved uninfected cells (data not shown). When values of uninfected cells were subtracted from values of infected cells, substantial inductions of c-Jun, STAT1 α , MEF2, c-Myc, ATF-2, and c-Fos were observed (Fig. 11A). This activation was lowest for MEF-2 and highest for c-Fos and c-Jun, while similar levels of induction were observed for c-Myc, STAT1 α , and ATF-2 (Fig. 11A).

To determine the specificity of the above transcription factor

FIG. 11. Effect of ERK inhibitor U0126 on the activation of MAPK-dependent transcription factors. (A) Monitoring MAPK-regulated transcription factor activity. Nuclear extracts prepared from uninfected HFF cells and HFF cells infected with KSHV for 120 min were tested for the activity of MAPK-regulated transcription factors by incubating the nuclear extracts to the plate-immobilized oligonucleotides containing various transcription factor-specific sites, followed by ELISA with phosphorylation-specific antibodies to the respective transcription factors. For competition, soluble oligonucleotides containing various transcription factor-specific sites (wild type) or its mutant form were added to the nuclear extracts. Positive controls provided for each transcription factor were used simultaneously. The histogram represents the activation levels of c-Jun, STAT1 α , MEF2, c-Myc, ATF-2, and c-Fos in the nuclear extracts from KSHV-infected HFF cells. Data represent the average $\pm$ standard deviation of three experiments, and values shown here are after the subtraction of values from uninfected cells. (B) Histogram depicting the percent inhibition of DNA binding of MAPK-dependent transcription factors in nuclear extracts from HFF cells pretreated with U0126 and then infected with KSHV for different time points. Percent inhibition was calculated with respect to the DNA binding activities in KSHV-infected HFF cells without U0126 pretreatment. Data represent the average $\pm$ standard deviations of three experiments.

detection, uninfected and infected nuclear extracts were pre-incubated for 30 min with WT or mutated consensus sequences for the respective factors before the addition to the plate-bound WT DNA sequences. In this experiment, transcription factors prebound to the WT oligonucleotides will be unable to bind to the plate-bound DNA and, hence, will be removed in the washing steps. The ability of transcription factors to bind their respective target sequences was significantly inhibited by preincubation with WT oligonucleotides, while no effect was seen with mutated oligonucleotides (Fig. 11A). These results demonstrated the specificity of the assay used to detect the transcription factors induced by KSHV infection.

To analyze the effect of the ERK inhibitor U0126 in KSHV induction of transcription factors, HFF cells were pretreated with the drug and then infected with KSHV for 5, 15, 30, 60, and 120 min, and activities of various transcription factors in

the nuclear extracts of uninfected and infected cells were measured. Since the levels of these transcription factors were very low in the uninfected cells, we did not observe a significant effect with U0126 pretreatment. In contrast, when cells pretreated with U0126 were infected with KSHV, significant reductions in KSHV-induced transcription factors such as c-Jun, c-Fos, and c-Myc were observed (Fig. 11B). The DNA binding activity of c-Fos as well as c-Jun was inhibited considerably, with inhibition ranging from 12%, 18%, 20%, 52%, and 58% for c-Fos and 20%, 33%, 36%, 47%, and 62% for c-Jun at 5, 15, 30, 60, and 120 min p.i., respectively (Fig. 11B). The DNA binding activities of c-Myc was inhibited by 8%, 8.17%, 9%, 4%, and 39% (Fig. 11B) after 5, 15, 30, 60, and 120 min p.i., respectively, suggesting that activation of c-Myc is dependent on the phosphorylation of ERK. Only a moderate level of inhibition was seen for the DNA binding activity of STAT1 α

and ATF-2 (Fig. 11B). In contrast, no appreciable level of MEF2 activity was seen with U0126 pretreatment (Fig. 11B). These results suggested that KSHV-mediated ERK phosphorylation plays key roles in the activation of c-Fos, c-Jun, c-Myc, and STAT1 α transcription factors in the infected cells.

Nuclear extracts from KSHV-infected cells bind strongly to AP1 and RTA-AP1 sequences. AP1 is a family of regulatory proteins characterized as immediate-early inducible transcription factors that were shown to be activated by a variety of stimuli involved in numerous biological processes, including cellular and viral gene expression, cell proliferation, differentiation, and tumorigenesis. AP1 is not a single transcription factor; instead, it is the name given to a DNA binding activity specific for the palindromic sequence TGAGTCA. AP1 is composed of a mixture of heterodimeric complexes of proteins derived from the Fos and Jun families including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD. Primarily, AP1 dimers bind to DNA on a TPA-response element with the 5'-TGA(C/G)TCA-3' sequence. Jun-Fos heterodimers form more stable complexes with TPA-response elements. These complexes display stronger transactivating activity than Jun-Jun homodimers and c-Jun/c-Jun homodimers, and c-Jun/c-Fos heterodimers preferentially bind to the AP-1 consensus sequence TGA(C/G)TCA (7). Only Jun proteins can form transcriptionally active homodimers within AP1 members or heterodimers with CREB/ATF members to bind the *cis*-acting replication element (5'-TGACGTCA-3'). The intracellular signaling cascades controlling AP1 activation are highly complex and may involve different kinases, and MAP kinases are one of the many potential upstream kinases involved in activation of AP1. ERK1 and ERK2 can directly phosphorylate the AP1 subunits Fos and Jun and the Ets family members Elk-1 and SAP1 α (12, 25, 26, 37, 48). RSK1 can directly phosphorylate SRF, Fos, and Jun (12).

Studies have demonstrated the ability of Jun and Fos to transactivate the KSHV ORF 50 (RTA) promoter through an AP1-binding site mapping at positions -87 to -81 (66). Since our studies suggested a role for KSHV infection-induced ERK1/2 in RTA expression and as well as the significant activation of transcription factors such as c-Fos and c-Jun that are known to induce RTA promoter, we analyzed the nuclear extracts of HFF cells or HFF cells pretreated with U0126 infected with KSHV for 60 and 120 min by EMSA with wild-type or a mutated AP1 site present in RTA promoter for the direct evidence of AP1 activity. Nuclear extracts from uninfected HFF cells did not show appreciable levels of AP1 binding (Fig. 12A, lane 2), whereas nuclear extracts from KSHV-infected cells could bind very efficiently to AP1 consensus probe (Fig. 12A, lane 3) as well as RTA-AP1 probe (Fig. 12A, lane 4). This binding was approximately sixfold higher than the AP1 activity observed in the uninfected cells (Fig. 12A, lane 2). To check the specificity of protein-DNA interaction, competitive EMSA was performed, in which a 100 times the molar excess of cold double-stranded AP1 (WT) or RTA-AP1 oligonucleotide probe was added as competitor for the respective treatments. No AP1 (Fig. 12A, lane 5) or RTA-AP1 (Fig. 12A, lane 6) binding to the target DNA was observed in the competitive assay. Similar results for AP1 binding as well as RTA-AP1 were obtained from HFF cells infected with KSHV for 120 min (data not shown). These studies demonstrated direct

evidence for the KSHV infection-induced AP1 activity and suggested a role in viral gene expression.

MEK1/2-ERK1/2 inhibition significantly inhibits the AP1 binding activity in KSHV-infected cells. Pretreatment of HFF cells with ERK inhibitor U0126 resulted in the significant inhibition of AP1 DNA binding for the AP1 consensus (Fig. 12A, lane 7) as well as RTA-AP1 probe (Fig. 12A, lane 8), with about 48 to 50% inhibition as determined by densitometry. A similar inhibitory effect for AP1 binding was also observed in the nuclear extracts from HFF cells pretreated at 37°C for 1 h with another MEK inhibitor, PD98059 (50 μ M) (data not shown). Pretreatment of HFF cells with ERK inhibitor U0126 and then infection with KSHV for 120 min also resulted in about 50% inhibition of AP1 DNA binding for the AP1 consensus as well as RTA-AP1 probe (data not shown). Nuclear extracts prepared from KSHV-infected cells did not bind either to AP1m (Fig. 12B, lane 3) or RTA-AP1m (Fig. 12B, lane 4). Similarly, no DNA binding to either AP1m or RTA-AP1m was seen in extracts prepared from U0126-treated and KSHV-infected HFF cells (Fig. 12B, lanes 5 and 6), respectively. These results demonstrated the specificity of protein-DNA interactions detected by EMSA. Taken together, these results suggest that KSHV-induced ERK plays a role in RTA induction by its ability to modulate transcription factors activating the RTA promoter.

DISCUSSION

To establish a successful infection, KSHV as well as other viruses needs to overcome several host cell obstacles such as apoptosis triggered by the engagement of multiple receptors during virus binding and entry processes, induction of innate and inflammatory immune responses including interferons activated by Toll and other receptors, cellular interference RNAs, autophagy, and restriction on virus gene transcription. It is imperative to effectively neutralize these obstacles very early during infection prior to *de novo* viral gene transcription. Owing to the requirement of a very rapid interference response and limited genome sizes, besides using their gene products, viruses have probably evolved better strategies to overcome these obstacles. Manipulation of host cell preexisting signal pathways via their interaction with cell surface receptors is probably one of the best strategies that fulfills these requirements. KSHV interacts with cell surface HS, α 3 β 1 integrin, and probably with other yet to be identified receptor(s). Within 5 min of target cell interactions, KSHV and KSHV-gB induced a rapid increase in PI 3-K activity, which is the well-known upstream effector for the host anti-apoptotic Akt pathway. Moreover, KSHV induction of FAK-Src and PI-3K are essential for viral DNA internalization (54), and induction of Rho-GTPases is essential for the migration of capsid in the cytoplasm and nuclear delivery (42). The present study shows that KSHV induces ERK1/2 very early in infection, probably allowing it to overcome the restriction on viral gene transcription imposed by the host cell and facilitate the viral gene expression and thus the establishment of infection. These studies further support our hypothesis that, beside the conduit for viral DNA delivery into the cytoplasm, KSHV interactions with α 3 β 1 integrin and other receptor(s) create an appropriate intracellular environment facilitating infection.

FIG. 12. Effect of ERK inhibitor U0126 on AP1 binding to the ORF50 promoter. (A) EMSA experiment showing binding of nuclear extracts prepared from virus-infected and uninfected HFF pretreated or untreated with ERK inhibitor U0126 to the wild-type AP1 as well as RTA-AP1. Specificity of the DNA-protein interaction was assessed by competition EMSA using a 100 times the molar excess of unlabeled double-stranded oligonucleotide AP1 probe (lane 5) and unlabeled RTA-AP1 (lane 6) as competitors for respective treatments. Each EMSA is representative of at least three independent experiments. The binding of AP1 in the uninfected cells was considered 1 for comparison and expressed as the increase in DNA binding (*n*-fold) in nuclear extracts prepared from virus-infected HFF cells. (B) EMSA experiment showing the binding of proteins present in the nuclear extracts prepared from HFF cells infected with KSHV, with and without pretreatment with ERK inhibitor U0126 to the mutated target sites of AP1 (AP1m) and RTA (RTA-AP1m). Each EMSA is representative of at least three independent experiments.

Early kinetics of the ERK1/2 and MEK1/2 signaling pathway, its activation by UV-inactivated KSHV in the absence of viral gene expression, and the rapid nuclear accumulation clearly demonstrated a role for virus binding and/or entry but not viral gene expression in this induction. The studies presented here demonstrate that activation of the MEK and ERK pathways plays a major role in viral infection at a postnuclear entry stage. The virus preparations used in our studies represent mostly enveloped KSHV virion particles (32, 44), and only enveloped virus particles were seen in the electron microscopy examinations, indicating the purity of virus preparations (2, 3, 5). Since every viral genome contains a single copy of the ORF 73 gene, the number of viral DNA molecules could be calculated from the corresponding copy numbers of the ORF 73

gene. Hence, we extracted the KSHV DNA from the purified virus, and copy numbers were quantitated by real-time DNA PCR using primers amplifying the KSHV ORF 73 gene. We routinely infected cells with 5 to 10 DNA copies per cell, which obviously represented both infectious and noninfectious enveloped virus particles. When infection was measured by GFP and/or ORF 73 expression (4, 32, 44), we observed >30% infection with 5 DNA copies per cell, which indicated the number of infectious virus particles. The ratio of particles to infectious particles varied from batch to batch. Even though a copy number of 5 to 10 DNA copies per cell appears to be low in terms of infectious particles that are capable of expressing viral genes, since our studies were examining the very early consequences of enveloped virus binding to the target cells,

namely, the induction of preexisting signal pathways prior to the viral gene expression, a mixed population of infectious and noninfectious virus particles does not alter the conclusions drawn from our experiments.

The ability of soluble gpK8.1A to induce a robust ERK1/2 response in HMVEC-d and HFF cells and of soluble gB to induce only a limited response in HMVEC-d suggests that gpK8.1A is the key mediator of ERK1/2 induction by KSHV. Our studies also clearly demonstrated a role for KSHV gpK8.1A-induced ERK1/2 in viral gene expression and, hence, in KSHV infection. This is in contrast to a recent work by Luna et al. (36) in which a K8.1-null recombinant virus (BAC36ΔK8.1) was constructed by deletion of most of the K8.1 ORF and insertion of a kanamycin resistance gene cassette within the K8.1 gene. Transfection of BAC36ΔK8.1 DNAs into 293 cells produced infectious virions in the supernatants of transfected cells. Based on their results, the authors concluded that gpK8.1A is not required for KSHV entry into 293 cells (36). However, there are several reasons to suggest that these conclusions may not fully explain the role of gpK8.1A in virus entry. For example, for infection in these studies, a 50-μl portion of infectious viral inoculum was mixed with polybrene at a final concentration of 5 μg/ml which was placed on 293 cells for 5 h, and infectivity was determined at 2 days p.i. by counting the number of GFP-expressing cells. Since polybrene is used to enhance the infectivity of various viruses by eliminating the need for receptors (33), the observed infection with BAC36ΔK8.1 virus does not rule out the role of gpK8.1A in KSHV in virus infectivity. The study by Luna et al. (36) offers a second example. When cells transiently transfected with wild-type BAC36 KSHV and BAC36ΔK8.1 were induced with TPA, estimation of genome copy numbers shows that, compared to wild-type BAC 36 virus, three times more BAC36ΔK8.1 virus was present. However, during infection of 293 cells, virus preparations were not adjusted to copy numbers. Hence the observed infection level that was twice greater with BAC36ΔK8.1 virus than WT BAC36KSHV (36) could actually be low if the copy numbers were adjusted to WT BAC36 KSHV. Further studies are in progress to define the role of KSHV gpK8.1A in virus egress and entry.

The ability of KSHV to bind the target cells at 4°C was inhibited by about 42%, 53%, and 77% when virus was preincubated at 4°C with 75, 150, and 300 μg of soluble gpK8.1A, respectively (64). This suggested a role for gpK8.1A in the binding step (64). However, in another study, when endothelial cells were incubated with 100 μg of soluble gpK8.1A at 37°C, infection was not blocked, suggesting that gpK8.1A may not be essential for the binding step (10). This discrepancy could be due to the differences in temperature at which cells were preincubated with soluble gpK8.1A. Since endocytosis of plasma membrane-bound gpK8.1A at 37°C could not be ruled out in the studies by Birkmann et al. (10), it is possible that sufficient gpK8.1A was not available to block the binding and entry of KSHV. A similar situation is observed in the herpes simplex virus (HSV) system, where binding to HS greatly enhanced the infection (55, 57, 70). Similar to KSHV, HSV glycoproteins gB and gC bind to HS. Importantly, though soluble HSV-1 gC competed with attachment of HSV-1, plaque formation was not inhibited by gC even at high concentrations. Since both HSV gB and gC bind to HS, HSV can bind and enter target

cells even at high concentrations of soluble gC. Hence the discrepancy between Wang et al. (64) and Birkmann et al. (10) could be also due to the ability of KSHV gB to bind target cell HS and other molecules, even cells that were incubated with gpK8.1A (3), suggesting that KSHV has evolved redundant mechanisms for the initial attachment step.

We have previously demonstrated the inhibition of KSHV-induced ERK1/2 activation by preincubating KSHV with anti-gB antibodies, although anti-gB antibodies did not block virus binding to the cells (44). The present study shows that soluble gB per se is unable to induce an appreciable ERK1/2 response. Preincubation of KSHV with soluble human α3β1 integrin blocked the ERK induction (44), and infection of CHO cells induced a moderate ERK1/2 response which was augmented by the transfection of a plasmid expressing human α3 integrin (44). Together, these studies clearly indicated that ERK signaling induced by KSHV is at least in part mediated via α3β1 integrin. Reduction of KSHV-induced ERK activity by anti-gB antibodies, ERK activation, and infection in FAK-positive Du17 cells, and their reduction in FAK-negative Du3 cells despite efficient virus binding, strongly indicate the involvement of a postattachment step of the virus entry pathway in the signal induction. Taken together, these studies suggest that the ability of KSHV to interact with integrin via its gB is probably a key step to the subsequent interaction of gpK8.1A with its receptor(s). The nature of the receptor(s) recognized by gpK8.1A that is involved in the signal cascade activation leading into ERK1/2 phosphorylation is not known at present. Further work using gpK8.1A-deficient KSHV is in progress to define the relationship between gpK8.1A and gB interactions with host cells.

Emerging evidence suggests that manipulation of the host cell's ERK signaling machinery early during infection is a common theme used by many viruses such as human cytomegalovirus (27), Simian virus 40 (56), human immunodeficiency virus type 1 (24, 72), and influenza virus (47) to regulate their gene expression early during infection. Activation of MAPK/ERK1/2 and protein kinase A have been shown to play crucial roles in vaccinia virus multiplication, and ERK1/2 kinase activation required the modulation of actin dynamics, microtubule polymerization, and tyrosine kinase phosphorylation (6). Similar to our studies, respiratory syncytial virus infection up-regulated the expression of proinflammatory mediator genes in bronchial epithelial cells via the activation of ERK1/2 within 5 min, and inhibition of ERK pathways significantly decreased respiratory syncytial virus infection, suggesting the involvement of ERK in viral gene expression (31). Hepatitis B viral protein HBx has also been shown to activate the mitogen-activated protein kinase and PI 3-kinase signal cascade, which further enhanced the activation of transcription factors such as AP1 and NF-κB (15). Moreover, an ERK-specific inhibitor could block HBx-induced ERK1/2 activation (15).

As demonstrated in the present and previous studies (32), immediately following infection of primary human endothelial and fibroblast cells, KSHV expressed the latency-associated genes and a limited number of lytic cycle genes. The factor(s) responsible for the initiation of latent and lytic cycle of KSHV during primary infection and factor(s) governing the outcome of infection are not known. While it is possible that, like HSV-1 VP16 protein, KSHV may carry virion-associated transactivat-

ing protein(s), the requirement of ERK1/2 in latency ORF 73 and lytic ORF 50 (RTA) gene transcription is an interesting finding. Phosphorylation and activation of ERK1/2 are blocked by U0126, a potent and specific inhibitor of MEK1/2, thus probably preventing downstream phosphorylation of a number of transcription factors such as Elk-1, c-Fos, c-Jun, ATF/CREB, Ets-2, NF- κ B, SRF, and components of AP1 complex by ERK1/2 (19). It is interesting that the KSHV ORF 73 promoter/enhancer region possesses several transcription factor binding motifs which include sites for c-Myc, c-Jun, IRF1/2, AP1, Sp-1, NF-1, Oct-6, AP2, Oct-1 and glucocorticoid receptor (51). Similarly, Oct-1, AP1, and cellular CCAAT/enhancer-binding protein and other transcription binding motifs are also present in the promoter/enhancer region of KSHV ORF 50 (66). A recent study demonstrated that the AP1 activity may be crucial for very early activation of the RTA and K8 promoters during KSHV lytic cycle. Our results demonstrating the induction of MAPK-regulated transcription factors and their inhibition by U0126, detection of AP1 binding to RTA promoter in the infected cell nuclei, and inhibition by U0126 suggest that ERK1/2 via the activation of AP1 and other preexisting transcription factors plays important roles in the activation of ORF 50 and ORF 73 genes. Thus, by inducing ERK and the subsequent transcription factors, KSHV must be overcoming the transcriptional block in quiescent cells. Data presented here show that KSHV-induced ERK1/2 also plays a role in K8 and v-IRF2 gene expression. Since these genes are dependent upon ORF 50 expression, it is not clear whether the observed effects were due to a direct role of ERK1/2. K5 is involved in the down-regulation of major histocompatibility complex class I, ICAM-1, and B7.2 molecules, and thus probably blocks the elimination of infected cells by cytotoxic T and NK cells (39, 60). ERK1/2 appears to have little role in K5 gene transcription, and this could be due to the fact that K5 is regulated independently of ORF 50 expression (46). Further work is essential to decipher the role of ERK1/2, AP1, c-Jun, and NF- κ B in KSHV gene expression and in the subsequent interplay between ORF 73 and ORF 50 and lytic cycle expression of other genes.

Besides its role in viral gene modulation, KSHV-induced ERK1/2 also plays some role in virus-induced host gene modulation. Early during infection, KSHV reprograms the host gene transcription, thereby probably regulating a variety of cellular processes like apoptosis, signaling, cell cycle regulation, transcription, inflammatory response, and angiogenesis, all of which may play important roles in KS biology and KS pathogenesis. It is probable that many of the induced host genes play important roles in the establishment of infection, immune evasion, anti-apoptosis, and overcoming other obstacles in the cells. Our studies show that KSHV-induced ERK1/2 plays roles in the induction of some of the host cell genes. Phosphorylated ERK increases the transcriptional activity of transcription factors such as Ets-1, Ets-2, c-Jun, c-Myc, and CREB (38, 71). These transcription factors induce the expression of several genes important for cell cycle progression including HB-EGF (71). KSHV infection induces the expression of HB-EGF, which was inhibited in U0126-treated cells. Ets-binding sequences have been identified in the promoter regions of HB-EGF (38), suggesting the control of this gene by the ERK signaling cascade at the transcriptional level. More-

over Ets-2 cooperates with AP1, and this heterodimer binds to the Ets/AP1 site on the promoter of HB-EGF and induces its expression (38), suggesting that activation of Ets-2 mediated by upstream ERK phosphorylation is critical for HB-EGF expression. DUSP5 hydrolyzes nuclear substrates phosphorylated on both tyrosine and serine/threonine residues and has a potential role in deactivation of mitogen- or stress-activated protein kinases (41). DUSP5 has been reported to be a direct target of p53 (61). By using a novel mechanism, p53 negatively regulates cell cycle progression by down-regulating MAPK (61). DUSP5 expression is up-regulated by KSHV in an ERK1/2-dependent manner, implying that KSHV induces the phosphatases as a way to check the intracellular pools of active ERKs that are induced early during infection, thus possibly regulating the establishment of latent infection.

In summary, our studies demonstrate that KSHV induces the ERK1/2 pathway early during infection and thus facilitates the initiation of viral gene expression and establishment of infection. Development of specific inhibitors to ERK1/2 has proven useful in the treatment of certain cancers, and a second-generation MEK inhibitor such as PD184352 is currently in clinical trials. Further analyses of pathways linking ERK1/2 to virus and host gene expression will provide strategies to influence KSHV gene expression and infection and the associated diseases.

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