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Identification and Characterization of the Orf49 Protein of Kaposi's Sarcoma-Associated Herpesvirus

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Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Kaposi's sarcoma is the most common neoplasm among human immunodeficiency virus-positive individuals. Like other herpesviruses, KSHV is able to establish a predominantly latent, life-long infection in its host. The KSHV lytic cycle can be triggered by a number of stimuli that induce the expression of the key lytic switch protein, the replication and transcription activator (RTA) encoded by Orf50. The expression of Rta is necessary and sufficient to trigger the full lytic program resulting in the ordered expression of viral proteins, release of viral progeny, and host cell death. We have characterized an unknown open reading frame, Orf49, which lies adjacent and in the opposite orientation to Orf50. Orf49 is expressed during the KSHV lytic cycle and shows early transcription kinetics. We have mapped the 5′ and 3′ ends of the unspliced Orf49 transcript, which encodes a 30-kDa protein that is localized to both the nucleus and the cytoplasm. Interestingly, we found that Orf49 was able to cooperate with Rta to activate several KSHV lytic promoters containing AP-1 sites. The Orf49-encoded protein was also able to induce transcriptional activation through c-Jun but not the ATF1, ATF2, or CREB transcription factor. We found that Orf49 could induce phosphorylation and activation of the transcription factor c-Jun, the Jun N-terminal kinase (JNK), and p38. Our data suggest that Orf49 functions to activate the JNK and p38 pathways during the KSHV lytic cycle.

Kaposi's sarcoma-Associated herpesvirus (KSHV) is etiologically linked to Kaposi's sarcoma (12, 17, 25), primary effusion lymphoma, and multicentric Castleman's disease (3, 4). Kaposi's sarcoma is the leading neoplasm in human immunodeficiency virus-positive individuals. KSHV is the most recently described human herpesvirus, identified in 1994 by Chang et al. (6) via representational difference analysis. A member of the gamma subfamily of herpesviruses, KSHV is also the only known human rhadinovirus.

Like other herpesviruses, KSHV can establish both latent and lytic infection in its host. The lytic program can be triggered by a number of stimuli that induce the expression of the lytic switch protein, the replication and transcription activator (RTA) encoded by Orf50 (38). The expression of RTA is necessary and sufficient to trigger the full lytic program (21, 22) resulting in the ordered expression of all viral proteins, release of viral progeny and host cell death. RTA/Orf50 initiates the lytic cascade by inducing the transcriptional activation of viral promoters such as PAN, ORF6 (single-stranded-DNA-binding protein), K1, K2 (viral interleukin-6), K5, K6 (viral MIP-1), K8 (MTA), K9 (viral IRF), ORF57 (MTA), ORF50 (RTA), thymidine kinase, K12 (kaposin), K14 (viral OX-2), and ORF74 (viral G protein-coupled receptor) (5, 7, 9, 10, 16, 20, 21, 33, 37, 39, 44). This wide range of promoter activation occurs through sequence-specific binding of Orf50 to DNA via RTA responsive elements (5, 9, 20, 37) and binding to cellular transcription factors such as C/EBP-α and RBPJ-κ (19, 41).

Another protein expressed in the lytic cascade is K8 or K-bZIP, a structural and positional homologue of Epstein-Barr virus ZTA. However, unlike Epstein-Barr virus ZTA, K-bZIP is unable to activate lytic transcription from latency (30). K-bZIP shows strong *trans*-repression of viral ORF50 (15) and cellular p53 (28) promoters. Sumoylation of K8 mediates its repression (14).

Studies on KSHV pathogenesis have been limited to latently infected primary effusion lymphoma-derived B-cell lines such as BCBL-1, BC-2, JSC-1, BC-3 and BC-1. In these cell lines, latency can be disrupted by the addition of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or the histone deacetylase inhibitor sodium butyrate (24, 32). However, the efficiency of reactivation as measured by viral particle production, is very low, peaking at approximately twenty percent (42). Moreover, studies of KSHV latency are complicated by the fact that, at any time, ~5% of these cells are undergoing spontaneous lytic reactivation.

In an effort to enhance our understanding of KSHV pathogenesis, we focused our attention on Orf49, an uncharacterized open reading frame in the lytic switch locus. Orf49 is located adjacent to Orf50 and on the opposite strand. The gene encoded by Orf49 shows limited sequence homology to the Epstein-Barr virus *BRRF1* gene. Epstein-Barr virus *BRRF1* lies in the same relative orientation as KSHV Orf49 and is subject to a similar splicing event that omits the

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BRRF1 sequence from the Epstein-Barr virus RTA transcript (34).

Here we describe the initial characterization of KSHV Orf49 and its encoded protein. We have identified the 5′ and 3′ ends of the Orf49 transcript using rapid amplification of cDNA ends (RACE) and have characterized its transcription kinetics. The Orf49-encoded protein by itself could not activate any of the KSHV promoters we tested by promoter reporter assays, but it was able to cooperate with KSHV Orf50 to activate a number of lytic promoters containing AP-1 sites. We also found that Orf49 can activate the c-Jun and the JNK/p38 pathways. We suggest that Orf49-mediated activation of the p38/JNK pathway during lytic reactivation plays an important role in KSHV viral replication.

MATERIALS AND METHODS

Cell culture. Cos-1, 293, and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin and streptomycin, and 1× Gluta-max. CV-1 cells were further supplemented with nonessential amino acids. Rhesus fibroblasts (RhF) were cultured in Dulbecco's modified Eagle's medium with 10% Cosmic calf serum, penicillin and streptomycin, and 1× Gluta-max. The BCBL-1 cell line was grown in RPMI 1640 medium with 10% fetal bovine serum, penicillin and streptomycin, and L-glutamine. The BC-1 cell line was further supplemented with sodium pyruvate. All cell lines were maintained at 37°C and 5% CO_2. BCBL-1 and BC-1 cells were induced into lytic reactivation by treating with 25 ng/ml phorbol-12-tetradecanoate-13-acetate or mock treated with the carrier dimethyl sulfoxide (DMSO). Transient transfections were carried out with the Superfect (QIAGEN) reagent according to the manufacturer's directions.

Plasmids. KSHV Orf49 was PCR amplified from the DNA of BCBL-1 cells induced with TPA for 48 h using primers 5′ GCGGCGGAATTCATGGACTAC AAGGACGACGACAAGACATCGAGAAGGCCCC and 3′ CGCCGCAGA TCTACCTTTTTATTGTATACTGAACAATGC. The 5′ primer added a Flag epitope to the PCR product, which was subsequently cloned into the pSG5 vector (Stratagene). The Orf50 cDNA plasmid was amplified with primers 5′ CGCCGC GAATTCATGGCGCAAGATGACAAGGGTAAGAAGCTTCGGCGGTCC and 3′ CGCCGCAGATCTTTAACACTTGTCGTCGTCGTCCTTGTAGTC GTCTCGGAAGTAATTACGCC, tagged with Flag at the C-terminal end and cloned into pSG5. The orf50p, Orf57p, K8p, vIL-6p and vPolymerase promoter reporter constructs have been described previously (16, 19–22) and encode a viral promoter, which drives expression of the firefly luciferase gene in the pGL3-Basic vector (Promega). Transcription factor plasmids, also described previously (13), encode Gal-4, ATF1, ATF2, CREB, or c-Jun fused to the Gal4 DNA-binding domain. The E1B-CAT reporter plasmid contains five copies of the Gal4 DNA-binding site upstream of the E1B TATA box and chloramphenicol acetyltransferase (CAT) gene.

5′ and 3′ rapid amplification of cDNA ends. Total RNA from TPA-induced BCBL-1 cells was reverse transcribed using the Smart RACE cDNA amplification kit (Clonetech) with random hexamer primers. The product was used as template in a PCR with one of the following Orf49 specific primers (5′-1 CTT GACCAAAGCCGCGGAACCTAGGT, 5′-2 GGAATAAGCCAAATTCCGCCC TAGCCGC, 3′-1 TGTCGTTCAGATGTACCAGCGGTGCA, and 3′-2 GTCCGT CGCCACCCTCAATCCAGACT). 5′ and 3′ PCR products were cloned into pCR2.1-TOPO vector (Invitrogen) and individual colonies were sequenced.

RNA extractions and Northern blots. Total cellular RNA extractions were performed with the RNA STAT-60 reagent (Tel-Test) according to the manufacturer's instructions. Ten micrograms total RNA per sample was electrophoresed in 1.5% agarose-formaldehyde gels and transferred to nitrocellulose membranes (Hybond N+; Amersham Pharmacia) by capillary transfer for 16 to 20 h. Radioactive probes were made by random-primed oligonucleotide labeling (Random Primed DNA-labeling kit, Roche) of gel-purified PCR products with [α-^{32}P]dCTP (GE Healthcare). All hybridizations were performed using Quickhyb solution (Stratagene) for 16 to 20 h at 60°C in a rotating hybridization oven. Membranes were washed twice in 2× SSC (1× SSC is 0.14 M NaCl plus 0.015 sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and once with 0.1× SSC–0.1% SDS for 30 min at 60°C. Membranes were then analyzed with a PhosphorImager (Molecular Dynamics).

Western blots. Transfected cells in 100-mm dishes were washed twice with phosphate-buffered saline (PBS) harvested with 200 μl radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1%NP-40, 50 mM Tris [pH 6.8], 0.5% sodium deoxycholate, 0.1% SDS) and freeze-thawed three times. Cell debris was spun down and the supernatant was quantitated by Bradford assay (Bio-Rad). Cell lysates were incubated with SDS gel loading buffer at 95°C for 5 min, on ice for 1 min, and submitted to SDS-polyacrylamide gel electrophoresis (8).

For glycosylation experiments tunicamycin (Sigma) was added to cells to a final concentration of 20 ng/ml or mock treated with an equal volume of carrier and harvested after 48 h for Western blot analysis. Phosphatase treatments of whole-cell lysates were performed following incubation with monoclonal anti-Flag antibody (Sigma) for 1 h at room temperature and immunoprecipitation for 16 h at 4°C with AG beads (Santa Cruz). Beads were spun down and resuspended in 100 μl NEB buffer 3 and three microliters of calf intestinal phosphatase (New England Biolabs) or PBS followed by incubation for 1 hour at 37°C. Electrophoresed gels were transferred to nitrocellulose membranes (Hybond, Amersham) in a semidry transfer apparatus (Bio-Rad) at constant 20 V for 1 h and blocked in 5% dry milk, 1% Tween 20 Tris-buffered saline (TBSt) for 1 h at room temperature.

Peroxidase-labeled antibody to Flag epitope (Sigma), total and phosphorylated c-Jun, β-actin (Cell Signaling), and total and phosphorylated JNK antibodies (Upstate) were used at 1:1.000 dilutions in TBSt or 5% milk–TBSt. Membranes were washed three times for 5 min and immunoblots were detected with the SuperSignal chemiluminescent substrate (Pierce). For fractionation assays transfected cells were washed twice with 1× PBS and trypsinized for 5 min at 37°C. Trypsin was inactivated by adding an equal volume of complete growth medium (5% serum) and kept on ice. Cells were washed with PBS, supernatants were removed and cells were resuspended in 500 μl cytosolic lysis buffer containing 5 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES, pH 8.0), 85 mM KCl, and 0.5%NP-40, followed by a 5-min incubation on ice. Nuclei were spun down and the cytosolic fraction was removed. The nuclear fraction was washed with PBS, spun down, and lysed with RIPA buffer. Finally, both fractions were freeze-thawed thrice and cleared by centrifugation.

Immunofluorescence assays. Cos-1, CV-1, and RhFs were seeded in six-well plates and transfected with 4 μg DNA per well. Forty-eight hours posttransfection cells were washed twice with 1× TBS and fixed in 1:1 methanol-acetone at room temperature for 1 min. Cells were washed four times with TBS and incubated with 1:1,000 anti-Flag Cy3 (Sigma) in TBS for 1 h at room temperature. 4′,6′-Diamidino-2-phenylindole (DAPI) in PBS was added for 10 min followed by two more washes in TBS. Fluorescence was assayed in a Zeiss Axiovert 200 inverted microscope.

Promoter reporter assays. Six-well plates seeded with CV-1 cells were cotransfected with 1.5 μg promoter reporter construct and 1.5 μg of pSG5, pSG5-Orf49, or pSG5-Orf50 cDNA. One microgram of β-galactosidase expression plasmid was cotransfected into each sample to normalize for transfection efficiency. Each sample was analyzed in duplicate in at least two experiments. Forty-eight hours posttransfection cells were washed once in cold 1× PBS and lysed by freeze-thawing in 1× reporter lysis buffer (Promega). Lysates were analyzed for luciferase activity with the luciferase assay system (Promega) and for β-galactosidase activity with the Galacto-Star kit (Tropix) in a FLUOstar plate reader (BMG labtech).

Chloramphenicol acetyltransferase enzyme-linked immunosorbent assays. CV-1 cells were seeded in six-well plates and cotransfected with 1.2 μg E1B-CAT, 1.2 μg of transcription factor reporter, and 1.2 μg pSG5 or pSG5-Orf49; 0.6 μg of β-galactosidase expression plasmid was added to each sample to normalize for transfection efficiency. Forty-eight hours posttransfection cells were washed three times in cold 1× PBS and lysed. Cell lysates were spun down and incubated with a CAT enzyme-linked immunosorbent assay (ELISA) kit (11) in Microwell plates according to the manufacturer's recommendations. CAT expression was assayed using a FLUOstar plate reader (BMG labtech).

RT-PCR. c-Jun N-terminal kinase (JNK) inhibitor SP600125 or p38 inhibitor SB202190 (Sigma Aldrich) was added to a final concentration of 20 μM to BCBL-1 cells and simultaneously induced with TPA or mock induced with DMSO. RNA was harvested 24 h postinduction using the RNA STAT-60 reagent (Tel-Test) according to the manufacturer's instructions and equal amounts were reverse transcribed with the reverse transcription system kit (Promega). The product was diluted 10- to 50-fold and used as the template for PCRs with primers to viral GPCR, Orf57 (MTA), Orf49, LANA, and cellular glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

Identification of the Orf49 transcript. In an effort to identify the Orf49 transcript we performed 5′ and 3′ rapid amplifica-

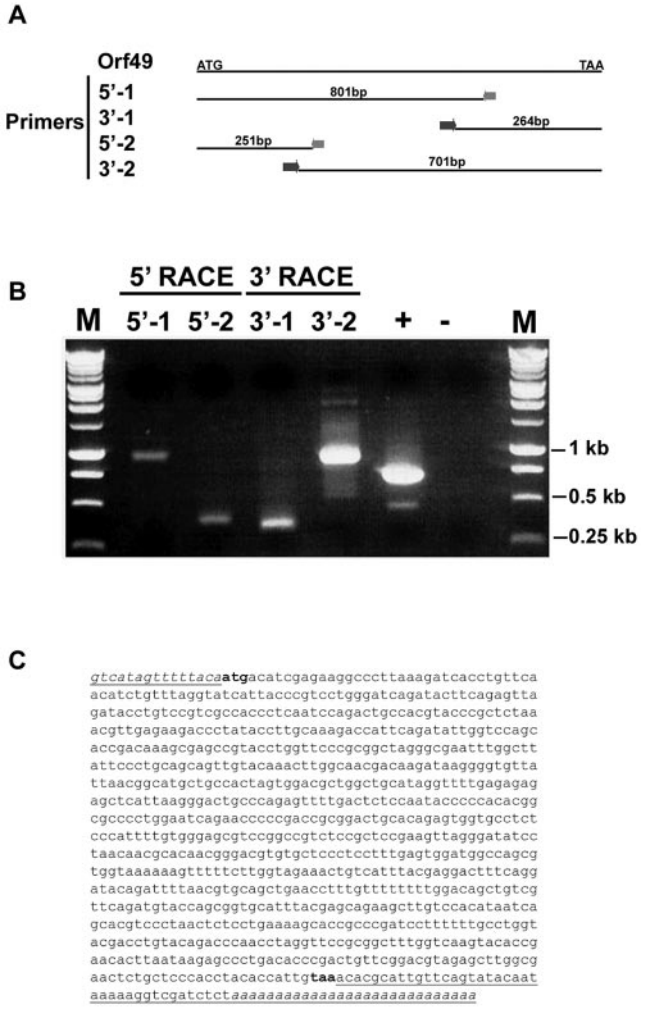

FIG. 1. Delineation of the full-length Orf49 transcript. A) RT-PCR design. Schematic representation of the Orf49 coding region (top) and location of RT-PCR primers designed for 5′ and 3′ RACE (arrowheads). B) Molecular cloning of RT-PCR products. Ethidium bromide visualization of RT-PCR products. Marker band sizes are provided on the right. Lanes 5′-1 and 5′-2 yielded products of ~900 and ~350 base pairs, respectively. Lanes 3′-1 and 3′-2 yielded products of ~340 and ~850 bp, respectively. Lane M, markers. Lane +, primers 5′-1 and 3′-2 yield an internal fragment of 750 bp. Lane −, negative control (no reverse transcriptase). C) Sequence of the full-length Orf49 transcript with 5′ and 3′ UTRs. RT-PCR products were cloned, sequenced, and aligned. A short 5′ UTR and a 3′ poly(A) tail are underlined. The transcript is not spliced and therefore identical to the genomic coding sequence.

tion of cDNA ends. Total RNA was isolated from TPA-induced BCBL-1 cells and used as the template for an initial reverse transcription step. Gene-specific primers were then used to amplify, clone, and sequence Orf49 cDNA. We designed Orf49-specific primer sets to amplify the 5′ and 3′ ends of Orf49 cDNAs and produce a 200-bp region of overlap between them enabling the alignment of the 5′ and 3′ clone sequences (Fig. 1A). The PCR products (Fig. 1B) were cloned into the TOPO 2.1 vector (Invitrogen). Since the length of Orf49 is about 930 bp, we were able to produce sequence alignments of the full-length Orf49 transcript including a short

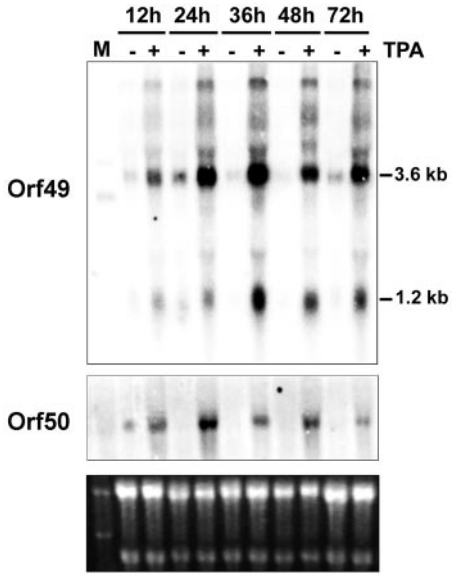

FIG. 2. Expression kinetics of Orf49. BCBL-1 cells were induced into lytic reactivation with 25 ng/ml TPA or mock treated with DMSO. Total RNA was harvested at 12, 24, 36, 48, and 72 h postinduction and 10 μg RNA was loaded per well of a formaldehyde-agarose gel. Northern blot analysis with an Orf49-specific probe reveals two Orf49 transcripts (upper panel), a longer, 3.6-kb transcript and a shorter 1.2-kb species. Middle panel: Northern blot with an Orf50-specific probe to demonstrate reactivation. Lower panel: loading control as visualized by ethidium bromide staining of the formaldehyde-agarose gel prior to transfer onto the nitrocellulose membrane.

5′ untranslated region (UTR) and the 3′ polyadenosine tail (Fig. 1C). As depicted in Fig. 1C, the full-length Orf49 transcript is not spliced and the coding sequence is identical to the genomic open reading frame.

Kinetics of expression of Orf49. In order to map the expression kinetics of Orf49 we induced BCBL-1 cells with 25 ng/ml TPA or vehicle (DMSO) and harvested total RNA at 12, 24, 36, 48, and 72 h postinduction. Ten micrograms of total RNA per sample was electrophoresed on an agarose-formaldehyde gel and the resolved RNAs were transferred to nitrocellulose. Northern blot analysis was performed with an Orf49 probe. Figure 2 (upper panel) shows that Orf49 RNA is only transcribed in TPA-induced cells and not in the DMSO-treated cells. Orf49 was observed in two different transcripts, 3.6 and 1.2 kb in size. The 1.2-kb transcript is very close in size to the predicted size of Orf49 while the 3.6-kb band likely represents a polycistronic transcript. The Orf49 transcripts were readily detectable at 12 h postinduction, indicating that this gene is transcribed with early kinetics. The signal peaked between 24 and 36 h postinduction and remained high at 48 and 72 h. A Northern blot with the Orf50-specific probe was included (middle panel) in order to demonstrate efficient reactivation. The lower panel in Fig. 2 shows the agarose gel with equal loading of the individual RNA samples. Orf49 thus encodes a lytic protein that is transcribed with early kinetics and to high levels.

Characterization of the Orf49 protein. The cDNA for KSHV Orf49 was PCR amplified and a Flag epitope was inserted at the N terminus of the protein. The PCR product was then

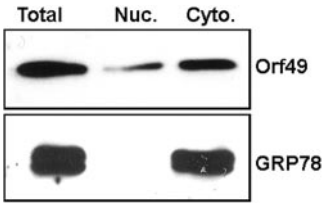

FIG. 3. Cell fractionation of Orf49-transfected cells. Cos-1 cells were transfected with Orf49-Flag and separated into nuclear and cytoplasmic fractions. Equal amounts of protein were loaded onto SDS-PAGE gels and transferred to nitrocellulose and the blots were probed with Flag antibody. The upper panel shows Orf49-Flag expression in the nucleus (Nuc.) and the cytoplasm (Cyto.). The lower panel shows expression of GRP-78, a bona fide cytoplasmic marker.

cloned into the pSG5 (Stratagene) vector and transfected into Cos-1 cells. Orf49 ran as a 30-kDa protein on sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE). Sequence analyses of Orf49 suggested several possible N-linked glycosylation and phosphorylation sites but no significant similarity to other known proteins or cellular localization signals. The glycosylation state of Orf49 was determined by treating cells with tunicamycin, an inhibitor of N-glycosylation, for 48 h and performing an Orf49 Western blot. Tunicamycin treatment did not change the electrophoretic profile compared to mock treatment, suggesting that Orf49 is not N-glycosylated (data not shown).

In order to identify the phosphorylation state of the Orf49 protein we transiently transfected Cos-1 cells with the Orf49-Flag plasmid and immunoprecipitated the lysates with anti-Flag monoclonal antibody. Lysates were either treated with phosphatase or mock treated with PBS followed by Western blotting with anti-Flag antibody. There was no difference in migration between phosphatase-treated versus mock-treated cell lysates, suggesting that the protein may not be phosphorylated (data not shown). However it is possible that if phosphorylated, the protein is either resistant to calf intestinal phosphatase treatment or the mobility shift is too small to be detected by conventional SDS-PAGE.

We next determined the intracellular localization of the Orf49-encoded protein. Cos-1 cells and rhesus fibroblasts were transfected or mock transfected with Orf49 plasmid. Cos-1 cells were harvested using a cell fractionation method in order to separate the cytoplasmic and nuclear fractions of lysates. The nuclear and cytoplasmic samples and a nonfractionated, total protein sample were subjected to Western blot with Flag antibody. As shown in Fig. 3, the Flag-tagged Orf49 protein is detectable in the nucleus of Cos-1 cells as well as in the cytoplasm. We used the cytoplasmic marker GRP-78 (35) as a control for the stringency of the cell fractionation method to show that there was no detectable cytoplasmic contamination in the nuclear fraction.

For immunofluorescence we chose rhesus fibroblast (RhF) cells because of their elongated shape and clear demarcation of the nucleus and cytoplasm. At 48 h posttransfection RhFs were subjected to immunofluorescence assays using an anti-Flag antibody conjugated to Cy3 dye and DAPI, a nuclear stain. We found that Orf49 was present in both the cytoplasm and nucleus. Figure 4 shows immunofluorescence of Orf49 in the RhF cells. Panels a and e show bright-field images of the cells, panels b and f represent Orf49 staining, while panels c and g represent nuclear staining with DAPI. A merged view of Orf49 and nuclear staining is shown in panels d and h. We observed similar cellular localization profiles for Orf49 in CV-1 and 293 cells (data not shown).

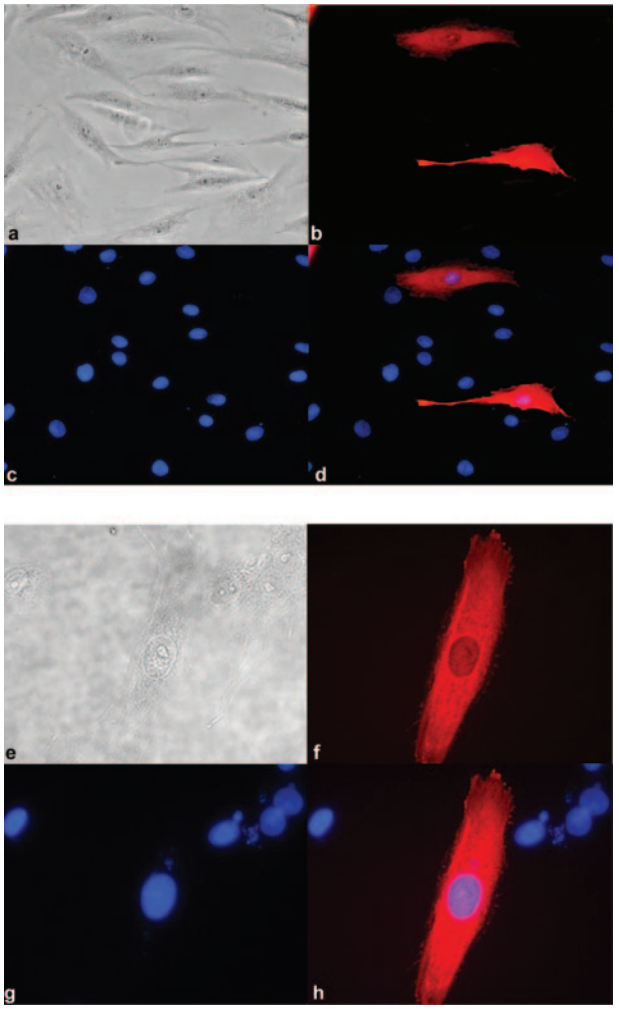

FIG. 4. Cellular localization of Orf49. Rhesus fibroblasts were transfected with an expression vector encoding a Flag-tagged Orf49 protein and 48 h later, cells were fixed and stained with the nuclear DAPI stain (blue) and a Cy3-conjugated antibody to the Flag epitope (red). Orf49 is readily detected in both the nucleus and cytoplasm of the cell. Panels a and e: phase contrast; panels b and f: Cy3; panels c and g: DAPI; panels d and h: merged DAPI and Cy3 stains. Panels a to d, 40× magnification; panels e to h, 100× magnification.

Orf50-mediated activation of key lytic promoters is enhanced by Orf49. Since Orf49 was expressed with early kinetics and appeared to be both nuclear and cytoplasmic, we attempted to determine if it could activate expression from a number of KSHV lytic promoters. We cotransfected a set of promoter-luciferase reporter constructs including Orf57p, viral G protein-coupled receptor promoter (vGPCRp), and K8p with cDNA expression plasmids for either Orf50, Orf49 or both. Although Orf49 was in itself unable to achieve high levels of activation of any of the promoters tested, we observed a cooperative effect of Orf49 with Orf50 on the activation of

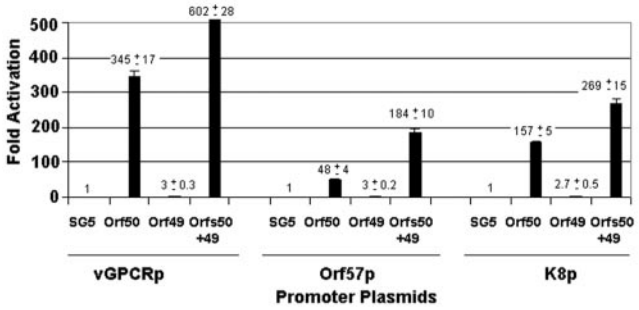

FIG. 5. Orf49 enhances Orf50-mediated induction of K8, Orf57, and vGPCR lytic promoters. Promoter reporter assays: empty vector (pSG5), Orf50, Orf49, or Orf50 plus Orf49 expression constructs were cotransfected into CV-1 cells with plasmids in which the vGPCR, K8, and Orf57 lytic promoters drive luciferase expression and 48 h post-transfection, promoter activity was determined by luciferase assay. Luciferase activities were normalized to β-galactosidase and expressed as fold induction over empty plasmid. Fold activation values are indicated above each bar along with the standard error. Each experiment was performed in duplicate and the experiment was repeated several times.

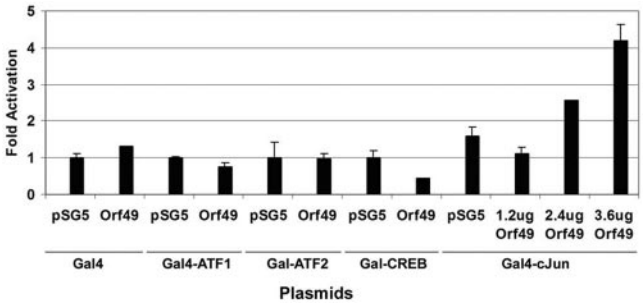

FIG. 6. Orf49 enhances c-Jun transcriptional function. pSG5 empty vector or Orf49 expression plasmids were cotransfected into CV-1 cells with plasmids encoding five copies of the Gal4 binding site upstream of the CAT gene and Gal4 protein fusions of ATF1, ATF2, CREB, and c-Jun. Orf49 enhanced c-Jun-mediated transcription in a dose-dependent manner but did not affect ATF-1, ATF-2-, or CREB-dependent transcription function.

Orf57p, vGPCRp, and K8p (Fig. 5). Orf49 could augment Orf50-mediated transactivation of these promoters.

It is important to note that Orf50-mediated induction of these promoters ranged from 50- to 300-fold over that of empty plasmid, arguing for a strong enhancing effect by Orf49 over an already high level of expression. (Fig. 5). There was no cooperation with Orf50 on the induction of the viral interleukin-6, Orf50, Pan (nut-1), and viral DNA polymerase promoters by Orf49 (data not shown). Closer inspection of the promoter sequences cooperatively activated by Orf50 and Orf49 revealed that they all contain AP-1 sites, raising the possibility that Orf49 may augment Orf50 transcription in an AP-1-dependent manner.

Orf49 can enhance c-Jun transcriptional function. In order to determine if Orf49 could activate transcription factors through the activation of AP-1 sites, we performed chloramphenicol acetyltransferase reporter assays with a c-Jun reporter plasmid. We examined whether Orf49 could augment the function of ATF-1, ATF-2, CREB, or c-Jun fusion proteins linked to the Gal4 DNA binding domain. Orf49 was cotransfected with ATF-1, ATF-2, CREB-1, or c-Jun Gal4 protein fusion expression plasmids into CV-1 cells, and the activation of a reporter plasmid containing five copies of the Gal4 DNA binding site upstream of the CAT gene was measured by a CAT ELISA.

As shown in Fig. 6, Orf49 was unable to activate transcription through the ATF1, ATF2, and CREB transcription factors. However, there was a dose-dependent, ~5-fold induction of the c-Jun transcription factor over that of empty plasmid. Since the DNA binding activity in this experiment was mediated through the Gal4 DNA binding domain of the individual fusion proteins, these results suggest that Orf49 activates promoters by enhancing c-Jun transcriptional function. Moreover, the selective activation of c-Jun but not other transcription factors could explain why Orf49 was able to enhance Orf50 induction of only those promoters with AP-1 sites, given that c-Jun is one of the strongest activators of AP-1 elements.

Orf49 is capable of inducing c-Jun phosphorylation. The activation of c-Jun is attributed to the phosphorylation of serine residues 63 and 73 (31, 36). In order to confirm that c-Jun was activated in cells expressing Orf49, we transfected CV-1 cells with an Orf49 expression plasmid and harvested protein lysates in the presence of phosphatase inhibitors. Western blot analyses were performed using antibodies against total c-Jun or c-Jun phosphorylated at both serine residues 63 and 73 to determine the phosphorylation and activation state of c-Jun in the presence and absence of Orf49. Figure 7A shows that c-Jun phosphorylation is greatly increased in the presence of Orf49 compared to the empty-vector control.

c-Jun phosphorylation is mediated by c-Jun N-terminal kinase. We transfected 293 cells with an Orf49 expression plasmid and performed Western blots to determine if JNK kinase was activated in the presence of Orf49. JNK activation can be measured by the phosphorylation status of threonine 183 and tyrosine 185 residues (18). We found that Orf49 was in fact able to induce the phosphorylation of JNK in 293 cells (Fig. 7B), leading to the activation of c-Jun and c-Jun-mediated transcription. In addition, p38 kinase, another member of the mitogen-activated protein (MAP) kinase family, was also phosphorylated and activated in Orf49-expressing cells compared to the vector control (Fig. 7B).

In order to ascertain if JNK and p38 are indeed activated in the more physiologically relevant context of reactivation of latently infected B cells, we used a BCBL-1 cell line in which the lytic switch RTA is inducible by tetracycline (26). In these cells RTA expression is tightly regulated by tetracycline (or doxycycline) and upon induction follows the strict pattern of gene expression characteristic of herpesviruses. Western blots for phospho-JNK and phospho-p38 were performed on lysates from the RTA-TREX BCBL-1 cells and the negative control pcDNA-TREX BCBL-1 cells, with or without a 24-h doxycycline induction (final concentration of 1 μg/ml). Western blots for RTA were also performed to demonstrate the doxycycline-mediated induction of RTA. As shown in Fig. 7C, both JNK and p38 are activated after induction of RTA-TREX BCBL-1 cells but not pcDNA-TREX BCBL-1 cells, thus demonstrating that RTA-induced lytic reactivation does induce phosphorylation/activation of JNK and p38 kinases and that

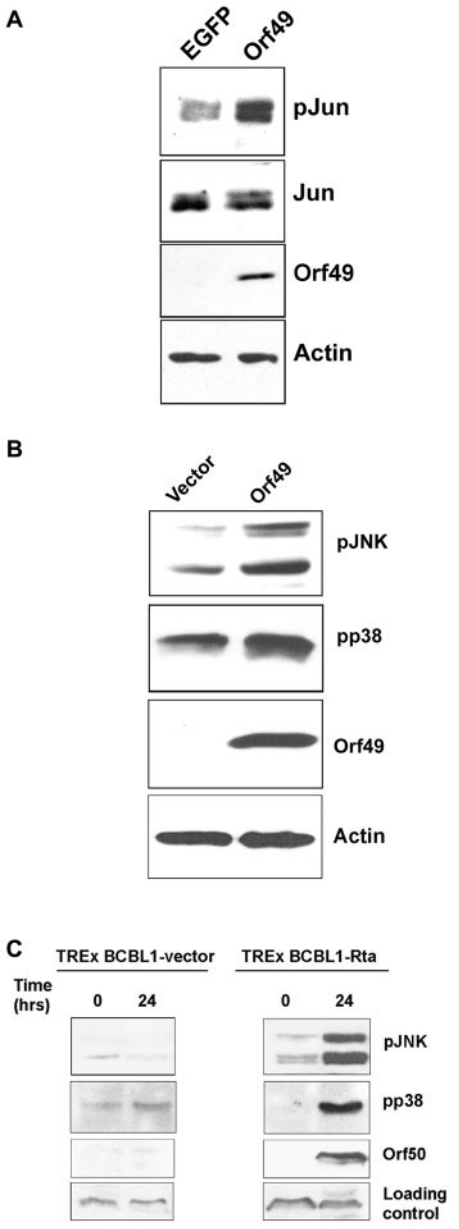

FIG. 7. Orf49 induces phosphorylation of c-Jun by activation of JNK and p38. A) CV-1 cells were transfected with the enhanced green fluorescent protein (EGFP)vector or an Orf49 expression plasmid and cells were harvested and lysed 48 h posttransfection. Phospho-c-Jun levels increased in the presence of Orf49 (first panel) while total c-Jun levels (second panel) remained comparable. The third panel shows expression of Orf49. Actin expression is also shown in the bottom panel to indicate equivalent loading. B) Both JNK and p38 pathways are activated by Orf49. 293 cells were transfected with pSG5 vector or Orf49 expression plasmids, serum starved for 24 h, and harvested 48 h posttransfection. Lysates were subjected to Western blot analysis with anti-phospho-JNK (pJNK) and anti-phospho-p38 (pp38) antibodies. Cells expressing Orf49 (as determined by Western blot with anti-Flag) showed vastly increased levels of phospho-JNK and phospho-p38 compared to the empty-vector control. Actin expression is also shown in the bottom panel to indicate equivalent loading. C) JNK and p38 are activated during reactivation. pcDNA-TREX-BCBL-1 cells carrying pcDNA (empty) vector or RTA-TREX-BCBL-1 cells carrying a doxycycline-inducible RTA were treated with 1 μg/ml doxycycline or untreated for 24 h. Lysates were harvested and Western blots for phospho-JNK, phospho-p38, and Orf50 were performed. Doxycycline-induced RTA-TREX BCBL-1s showed increased levels of both phospho-JNK and phospho-p38, while pcDNA-TREX BCBL-1 cells showed no activation of JNK or p38. Orf50 expression was only induced in the RTA-TREX BCBL-1 cells as expected.

our results are not an artifact of TPA-induction or transient transfection.

Taken together these results define the mechanism by which the Orf49 protein activates c-Jun-mediated transcriptional activation, by eliciting the phosphorylation and activation of JNK/p38 MAP kinases.

JNK/p38 activation is necessary for the expression of lytic proteins. In order to determine the effect of JNK/p38 on KSHV lytic reactivation and replication, BCBL-1 cells were induced with TPA and simultaneously treated with the JNK inhibitor SP600125 or p38 inhibitor SB202190. Total RNA was harvested at 24 h postinduction and reverse transcribed. The reverse transcription product was then diluted 10- to 50-fold and used as the template in a PCR with primer sets for the early lytic genes vGPCR, Orf57, and Orf49 as well as KSHV LANA and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) controls. Figure 8A shows the PCR products from these reactions. Both inhibitors seemed to decrease the levels of lytic expression of the vGPCR and Orf57 transcripts. The Orf49 transcript was unchanged in the presence of inhibitors, suggesting that Orf49 expression is not refractory to JNK and p38 inhibition. The LANA and GAPDH controls were stable regardless of treatment or replicative state.

We also performed Western blots to examine the expression of late lytic proteins. BC-1 cell lysates were harvested 24 h after treatment and equal amounts of protein were analyzed by Western blot with anti-KSHV Orf62 antibody. Orf62 is also called minor capsid protein or TRI-1 (27). As shown in Fig. 8B, treatment with both JNK and p38 inhibitors suppressed lytic reactivation as measured by expression of Orf62 protein. In summary, these results support a functional role for the JNK and p38 pathways in lytic reactivation.

DISCUSSION

We report here the first characterization of KSHV Orf49 located between the exons coding for Orf50 and on the opposite strand. We performed 5′ and 3′ RACE to identify the ends of the Orf49 transcript and found it to be unspliced. The Orf49 transcript was ~1,000 base pairs long, containing the expected coding sequence of over 900 bases plus a 15-nucleotide 5′ UTR and a 67-nucleotide 3′ UTR including the poly(A) tail. Northern blot analyses revealed that Orf49 transcripts were highly expressed during lytic reactivation with early kinetics and throughout the length of TPA treatment. Two transcripts were detected with an Orf49-specific probe, one of 1.2 and a second of 3.6 kb. The 1.2-kb transcript corresponds closely to our full-length cDNA clone. However, it is possible that there are longer, possibly bicistronic, transcripts that we did not detect via RACE. Expression levels peaked at 36 h post-TPA induction by Northern blot analyses. These results are similar to those reported by others (29; Dirk Dittmer, personal commu-

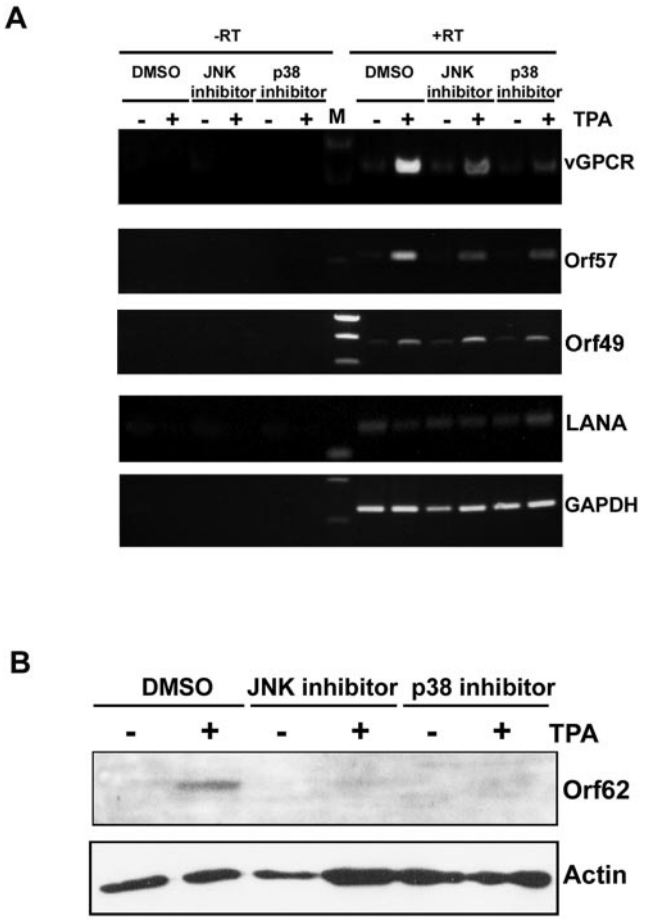

FIG. 8. JNK and p38 activation is necessary for lytic gene expression. A) Reverse transcription-PCR of total RNAs from TPA-induced or mock-treated BCBL-1 cells treated for 24 h with the JNK inhibitor SP600125 or the p38 inhibitor SB202190 was performed. PCRs using vGPCR and Orf57 primers revealed that expression levels of these early genes are inhibited by JNK and p38 inhibitors. However, expression of the Orf49 gene product was not inhibited by the presence of the JNK or p38 inhibitor. In addition, levels of the latency gene LANA were not affected by the JNK or p38 inhibitor. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was used as a loading control. B) JNK and p38 inhibition hinders late protein synthesis. BC-1 cells were induced with TPA (or mock induced with DMSO) and the JNK or p38 inhibitor. Cell lysates were harvested and equal amounts of total protein were analyzed by Western blots using antibody directed against the late protein Orf62/minor capsid protein. The bottom panel shows an actin loading control.

nication), who found that Orf49 expression peaks between 24 and 48 h using microarray analyses.

The cDNA encoded by Orf49 expressed a protein of ~30 kDa. We determined that the Orf49-encoded protein is not N-glycosylated or phosphorylated despite the presence of several possible glycosylation and phosphorylation sites in the coding sequence. However it is possible that the protein can be phosphorylated but is resistant to calf intestinal phosphatase. Additionally, the Orf49 protein was localized to both the nucleus and the cytoplasm of cells as observed by immunofluorescence and cell fractionation Western blots.

We next assessed the role of Orf49 in viral transcription. We used promoter reporter assays to evaluate if Orf49 was in fact able to induce expression from lytic viral promoters. Our results showed that Orf49 itself was not a potent transactivator but was able to cooperate with Orf50 to increase Orf50-mediated activation of the K8, Orf57 and vGPCR promoters. It is important to note that the activation of these promoters by Orf50 alone was already high ranging from 50-fold (Orf57) to 350-fold (vGPCR) over that of the empty vector. This strong activation of Orf57, vGPCR, and K8 suggests that Orf49 synergizes with Orf50 to increase the efficiency of lytic reactivation. Indeed, Orf57 has been shown to bind Orf50 and induce expression from the Orf50 promoter (23), suggesting that, in vivo, induction of Orf57 could indirectly enhance Orf50 expression and thus lytic reactivation.

The K8 protein binds the oriLyt and is essential for lytic replication (1), and hence, the increase in K-bZip expression mediated by Orf49 could significantly enhance the efficiency of lytic reactivation initiated by Orf50. Finally, the constitutively activated viral G protein-coupled receptor is an early lytic gene that plays important roles in angiogenesis. Thus, cooperative induction of these three viral promoters by Orf49 and Orf50 has tangible biological consequences for host cells undergoing lytic replication as well as the surrounding environment.

We have also identified a mechanism by which Orf49 induces expression of K8, Orf57 and vGPCR. These promoters all share AP-1 sites in addition to RTA-responsive elements. Using CAT reporter assays we demonstrated that Orf49 can augment c-Jun-mediated transcriptional activation and that this activation was dose dependent. These results were further validated by assessing phospho-c-Jun levels in the presence and absence of Orf49. Expression of Orf49 increased phospho-c-Jun levels compared to that of the vector controls. Orf50 has been shown to increase the levels of total c-Jun in DG75 cells, however, it did not induce c-Jun phosphorylation (40). Together these findings could suggest a mechanism by which Orf50 increases total c-Jun protein and Orf49 induces phosphorylation and activation of c-Jun, thus enhancing the total c-Jun transcriptional activation potential in the cell.

Since c-Jun activation is dependent on c-Jun N-terminal kinase, we also performed anti-phospho-c-Jun N-terminal kinase Western blots and found that Orf49 can indeed activate JNK. These results could indicate that c-Jun-mediated transcriptional activation is induced by Orf49 through JNK activation. Interestingly, Orf49 also induced phosphorylation/activation of the p38 kinase. Both the JNK and p38 pathways are activated by stress, growth factors and cytokines. They are activated by the same mitogen-activated protein kinase kinase kinases (MAP3Ks), share the MAP2K MKK4, and are often coactivated (43). Signaling through both pathways can result in proliferation, differentiation, or apoptosis although p38 activation is also involved in development, inflammation, and stress responses.

KSHV vGPCR has also been shown to trigger the activation of JNK and p38 (2), underscoring the significance of these pathways during lytic replication. Because of the wide-ranging effects of these pathways we wanted to assess the contribution of each pathway to lytic reactivation. JNK and p38 inhibitors blocked TPA-induced expression of early genes such as vGPCR and Orf57 as well as late genes such as Orf62/minor capsid protein. There was a marked decrease in the total amount of Orf62/minor capsid protein detected in both JNK and p38

inhibitor-treated, lytically reactivated cell lysates, indicating that signaling through both pathways can influence the amount of late structural proteins produced.

KSHV Orf49 is the positional homologue of the Epstein-Barr virus *BRRF1* gene encoding the Na protein. Na has been shown to encode a 34-kDa protein with early kinetics that is induced by ZTA (34). Hong et al. reported that Epstein-Barr virus Na functions in the reactivation of latent Epstein-Barr virus by inducing lytic promoters and increasing the amount of viral progeny released (13). They further showed that the *BRRF1* promoter was induced by ZTA through a CRE motif (ZII) and that Na enhanced Epstein-Barr virus RTA's ability to induce ZTA, thus creating a self-reinforcing loop. Epstein-Barr virus Na was also shown to activate c-Jun-mediated transcription. These findings led to the identification of Na as a transcription factor that activates ZTA expression through the activation of c-Jun and cooperates with RTA (BRLF1) in reactivation of Epstein-Barr virus from latency. However, since KSHV K8, the Epstein-Barr virus ZTA homolog, cannot by itself reactivate the virus from latency, it seems likely that the main function of KSHV Orf49 is to augment Orf50 transactivation of a subset of early promoters.

Thus, Orf49 represents an important member in the lytic reactivation cascade that enhances transcription from key Orf50-responsive promoters through the transcriptional activation of c-Jun and also activates the JNK/p38 kinase pathways, which are necessary for KSHV reactivation from latency as well as viral replication.

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