α-, β-, and γ-Herpesviruses encode putative viral protein kinases. The herpes simplex virus UL13, varicella-zoster virus ORF47, and Epstein-Barr virus BGLF4 genes all show protein kinase domains in their protein sequences. Mutational analysis of these herpesviruses demonstrated that the viral kinase is important for optimal virus growth. Previous studies have shown that ORF36 of Kaposi's sarcoma herpesvirus (KSHV) has protein kinase activity and is autophosphorylated on serine. The gene for ORF36 is expressed during lytic growth of the virus and has been classified as a late gene. Inspection of the ORF36 sequence indicated potential motifs that could be involved in activation of cellular transcription factors. To analyze the function of ORF36, the cDNA for this viral gene was tagged with the FLAG epitope and inserted into expression vectors for mammalian cells. Transfection experiments in 293T and SLK cells demonstrated that expression of ORF36 resulted in phosphorylation of the c-Jun N-terminal kinase. Autophosphorylation of ORF36 is important for JNK activation because a mutation in the predicted catalytic domain of ORF36 blocked its ability to phosphorylate JNK. Western blot analysis, using phosphospecific antibodies, revealed that mitogen-activated kinases MKK4 and MKK7 were phosphorylated by ORF36 but not by the kinase-negative mutant. Binding experiments in transfected cells also demonstrated that both the wild type and kinase-negative mutant of ORF36 form a complex with JNK, MKK4, and MKK7. In addition, using a tetracycline-inducible Rta BCBL-1 cell line (TREx BCBL1-Rta), JNK was phosphorylated during lytic replication, and inhibition of JNK activation blocked late viral gene expression but not early viral gene expression. In summary, these studies demonstrate that KSHV ORF36 activates the JNK pathway; thus this cell signaling pathway may function in the KSHV life cycle by regulating viral and/or cellular transcription.

Based on molecular and biological properties, the Herpesviridae family has been subdivided into the α-herpesvirus (herpes simplex virus and varicella-zoster virus), β-herpesvirus (human cytomegalovirus), and γ-herpesvirus (Epstein-Barr virus (EBV)) subfamilies (1). These double-stranded DNA viruses can remain latent and persist as an episome, expressing a limited number of viral genes, and thereby establish a lifelong infection in the natural host. Immunosuppression or stress can lead to reactivation of latent virus to produce pathogenic manifestations; examples include Burkitt’s lymphoma, nasopharyngeal carcinoma, and Hodgkin’s disease associated with EBV (for a review, see Ref. 2) and Kaposi’s sarcoma, primary effusion lymphomas, and multicentric Castleman’s disease associated with KSHV (for reviews, see Refs. 3 and 4). Reactivation of these latent herpesviruses leads to sequentially regulated expression of viral genes that play an essential role in the replication and assembly of infectious virions (2–4).

During the lytic cycle of infection, herpesviruses express genes that are predicted to encode protein kinases (for a review, see Ref. 5). These protein kinases are conserved among the Herpesviridae family, and analysis of the amino acid sequence reveals motifs that are shared by mammalian serine/threonine protein kinases. Located within the catalytic region of these viral proteins are 11 conserved subdomains that are common to cellular protein kinases. Recent studies have revealed that mutation of a conserved lysine residue within subdomain II abolishes phosphorylation; this finding shows that this region is necessary for the catalytic activity of these protein kinases (6, 7). The protein kinases of herpesviruses appear to mimic the function of cellular protein kinases, including Cdc2 and cellular translation factor EF-1α, by phosphorylating the same amino acid residues on their cognate protein targets (5, 8). Furthermore, the viral protein kinases are associated with the virion and may promote dissociation and entry of the virus particle by phosphorylating the viral tegument proteins (9–12).

The importance of herpesvirus protein kinases for viral replication and disease has been investigated. Kinase-null mutants generated in α-, β-, and γ-herpesviruses demonstrate decreased replication in tissue culture (13–15). Decreased virulence of HSV and VZV kinase-null mutants has been shown in the mouse model (16–18). However, the protein kinase of VZV appears to be dispensable for the establishment of latency in rodent models (14, 18).

Studies on cells productively infected with α-, β-, and γ-herpesviruses have demonstrated that various members of the stress kinase pathway are activated. HSV activates JNK, p38,
and AP1 (19–21); cytomegalovirus activates p38 and JNK (22); and VZV activates AP1, Jun, Fos, and ATF-2 (23). For EBV, the latent membrane protein LMP2A regulates c-Jun through an extracellular signal-regulated kinase (24), and the immediate early proteins of EBV, BZLF1 and BRLF1, activate ATF-2, p38, and JNK (25). Recent studies have demonstrated the K15 latent membrane protein LMP2A regulates c-Jun through an 5'-AAAAGATCTGCCACCATGGATTA-3' (26). Reactivation of KSHV in BCBL-1 cells (B-lymphocytes latently infected with KSHV) induces transcription of the viral protein kinase (27). Understanding the cellular events that are regulated by the KSHV protein kinase may provide a basis for defining molecular mechanisms that control viral gene expression. Our studies demonstrate the ability of KSHV ORF36 to activate the JNK pathway and the cellular transcription factor c-Jun, and inhibition of JNK activation resulted in the inhibition of late KSHV viral gene expression.

EXPERIMENTAL PROCEDURES

Antibodies—Phosphospecific antibodies for SEK1/MMK7 (Thr-216), MK7 (Ser-271/Tyr-275), stress-activated protein kinase/JNK (Thr-180/Tyr-182) were obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-phosphothreonine and -phosphoserine were obtained from Zymed Laboratories Inc. MEK-4 (C-20), MEK-7 (H-160), JNK1 (full-length), c-Jun (N terminus), c-Myc, and tubulin (H-300) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 was obtained from Stratagene (La Jolla, CA). Antibodies to KSHV K5.1/AVB were obtained from Advanced Biotechnology Inc. (Columbia, MD).

Cloning and Plasmids—The pND vector for expression in mammalian cells (a generous gift from Dr. G. Rhode, University of California, Davis, CA) was used in the cloning of KSHV ORF36. cdNA was synthesized using RNA extracted from BCBL-1 (B-lymphocytes latently infected with KSHV) cells after 48 h of 12-O-tetradecanoylphorbol-13-acetate treatment. KSHV ORF36 was amplified by PCR using appropriate oligonucleotide primers (5'-AAAGAATCTGCACCATGGATTA-CCAGGATGAGCAGATACCCGCGGCTAACGAAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
pathway. Western blot analysis demonstrated that these target proteins were not phosphorylated by either the wild type ORF36 or kinase-negative mutant K108Q (Fig. 2).

**ORF36 Activates JNK via MKK4 and MKK7**—MKK4 and MKK7 function as non-redundant activators of JNK in vivo. Synergistic activation of MKK4 and MKK7 is required for optimal JNK activation. Maximal enzyme activity of JNK is achieved through phosphorylation of threonine 183 and tyrosine 185 with MKK4 having a preference for phosphorylating the tyrosine residue and MKK7 having a preference for the threonine residue (30–33). To determine whether the viral kinase utilizes MKK4 and MKK7 in the activation of JNK, lysates from transfected cells expressing ORF36 or the mutant K108Q were analyzed by Western blot with appropriate antibodies. Fig. 3 demonstrates that both MKK4 and MKK7 were activated by ORF36 but not by the kinase-negative mutant K108Q. These observations reveal that the phosphorylation of JNK by ORF36 was dependent on the activation of both MKK4 and MKK7, and autophosphorylation of ORF36 at serine 183 was important for this activity.

**Phosphorylation of c-Jun by ORF36 via JNK**—Activated JNK regulates transcription by phosphorylating c-Jun, ATF-2, and other transcription factors. JNK phosphorylates the serine residues 63 and 73 of the transcription factor c-Jun, which in turn binds to 12-O-tetradecanoylphorbol-13-acetate response elements and thereby increases c-Jun expression (34–37). To demonstrate whether KSHV ORF36 activation of JNK results in c-Jun phosphorylation, total cell lysates were analyzed by Western blot analysis for phosphorylation of c-Jun. Fig. 4 demonstrates that activation of JNK by KSHV ORF36 results in c-Jun phosphorylation; the kinase-negative mutant K108Q did not lead to phosphorylation of c-Jun.

**ORF36 Interacts with MKK4, MKK7, and JNK**—Immunoprecipitation studies demonstrated that scaffolding proteins, such as JIP-1/2 (JNK-interacting protein 1/2), JSAP-1 (JNK/ stress-activated protein kinase-associated protein-1), and β-arrestin-2, interact physically with their downstream targets. JIP-1 and JIP-2 form complexes with JNK, mixed lineage kinase, and MKK7 (38, 39), whereas JSAP-1 forms complexes with MEKK1, MKK4, and JNK (40). These enzyme complexes produce “MAP kinase modules,” which facilitate activation and enhance specificity of their respective target proteins (39). To assess the in vivo association of ORF36 with proteins in the JNK pathway, 293T cells were transfected with ORF36 or the mutant K108Q. Immunoprecipitation of total cell lysates with the FLAG, MKK4, MKK7, or JNK antibody demonstrated physical interaction of ORF36 with these cellular enzymes (Fig. 5). The kinase-negative mutant K108Q was also able to associate with MKK4, MKK7, and JNK. Thus, autophosphorylation of ORF36 was not important for association with MAP kinase modules and proceeded in a phosphorylation-independent manner.

**Activation of JNK during KSHV Reactivation**—The KSHV viral protein Rta encoded by ORF50 is a transcriptional activator and acts as a molecular switch for KSHV reactivation. Expression of Rta efficiently induces lytic replication in latently infected KSHV cell lines. The TREx BCB1-L-Rta cell line is a derivative of the BCB1-1 cell line (B-cell lymphoma cell line latently infected with KSHV) in which Rta is under the control of a tetracycline-inducible promoter. This cell line was demonstrated to fully induce lytic replication and produce infectious viral progeny in the presence of tetracycline. In addition, the TREx BCB1-L-Rta cell line was demonstrated to induce KSHV gene expression in a more powerful and efficient manner than
that induced by 12-O-tetradecanoylphorbol-13-acetate stimulation of BCBL-1 cells (41). Stimulation of TREx BCBL-1-Rta cells with doxycycline for 24 h stimulated the expression of Rta, and expression levels did not change with the addition of JNK inhibitor (JNKi) II or the JNKi negative inhibitor (Fig. 6). Anisomycin or untreated cells did not express Rta. The same cell lysates were used to determine the activation of JNK and c-Jun. Induction of lytic replication activated JNK and c-Jun. This activation was abrogated in the presence of JNKi II. Inhibition of JNK also prevented expression of K8.1 (a KSHV glycoprotein that serves as a marker for viral late gene expression) but not K-bZIP (an early lytic gene identified as a basic-leucine zipper protein) (Fig. 6).

**DISCUSSION**

**KSHV, a γ2 herpesvirus, encodes a serine protein kinase (27).** Our results support previously published data, using phosphoamino acid analysis, that this kinase is phosphorylated on serine (Fig. 1) (27). Autophosphorylation was abrogated by mutating the lysine residue in the catalytic subdomain II of ORF36 (Fig. 1). In this study, we established a mechanism by which the viral protein kinase activates the mitogen-activated stress kinase pathway. Using transient transfection experiments, JNK was activated by KSHV ORF36 but not by the kinase-negative mutant K108Q (Fig. 2). This finding indicates that autophosphorylation of ORF36 was important for JNK activation. The p44/42, MKK3/6, and p38 pathways were not activated by ORF36, showing specificity for JNK activation (Fig. 2). Both MKK4 and MKK7 act synergistically to activate JNK (30–32). Fig. 3 demonstrates that ORF36 activates MKK4 and MKK7. These findings imply that JNK activation by ORF36 is via phosphorylation of MKK4 and MKK7. Recent data show that scaffolding proteins, such as JIP-1/2 and JSAP-1, associate with MEKK1, mixed lineage kinase, MKK4, MKK7, and JNK, forming enzyme complexes and facilitating the activation of the mitogen/stress-activated protein kinase pathway (38–40). To demonstrate whether ORF36 associates with these complexes, immunoprecipitation experiments were performed with antibodies specific for MKK4, MKK7, or JNK. These studies showed that KSHV ORF36 was associated with MKK4, MKK7, and JNK and that this association was independent of the phosphorylation state of ORF36. The signifi-

**Fig. 3. Synergistic activation of MKK4 and MKK7 by KSHV ORF36.** Lysates of transfected SLK cells were analyzed by Western blot using antibodies that recognized the phosphorylated forms of MKK4 and MKK7 (see "Experimental Procedures"). ORF36 activated both MKK4 and MKK7 (lane 3), whereas the mutant K108Q was unable to activate either MKK4 or MKK7 (lane 4). These results demonstrate that synergistic activation of MKK4 and MKK7 was important for the activation of JNK, and this activation was dependent on the phosphorylation state of ORF36 (lane 4). Equal loading of lysates was confirmed using antibodies that recognized total cellular MKK4 and MKK7 proteins. pMKK, phospho-MKK; Aniso, anisomycin.

**Fig. 4. Phosphorylation of c-Jun transcription factor by KSHV ORF36.** Activation of JNK results in the phosphorylation and activation of its substrate, c-Jun. To demonstrate whether JNK was catalytically active, phosphorylation of c-Jun was demonstrated in cells transfected with ORF36 (lane 3) but not in cells transfected with the mutant K108Q (lane 4) by Western blot analysis using anti-phospho-c-Jun antibody. Lane 1 (negative control) and lane 2 (positive control, anisomycin (Aniso)) served as controls for c-Jun phosphorylation. Equal loading of protein lysates was demonstrated by Western blot analysis using anti-c-Jun antibody. p-, phospho-

**cance of ORF36 activation of JNK is attributed to the phosphorylation and activation of the transcription factor c-Jun. In addition, we demonstrated that inhibiting the activation of JNK during KSHV reactivation severely represses expression of the late lytic viral gene K8.1 but had no effect on the expression level of the early lytic gene K-bZIP (Fig. 6). This demonstrates that initiation of lytic infection and the transcription of early lytic genes may not require the activation of the JNK pathway. Conversely expression levels of K8.1, a structural glycoprotein component of KSHV particles, are dramatically reduced in the presence of SP600125.

The physiological role of ORF36 for KSHV replication is not defined, but possible clues to its function have emerged from studies performed on homologous protein kinases found in other herpesviruses. Previous work demonstrated that α-, β-, and γ-herpesviruses encode viral protein kinases, which are represented by HSV-1 UL13, VZV ORF47, human cytomegalovirus UL97, human herpesvirus-6 U69, EBV EGLF4, KSHV ORF36, and rhesus rhadinovirus ORF36 (5, 42, 43). Similar to cellular serine/threonine protein kinases, these viral kinases contain 11 subdomains; a conserved lysine residue in subdomain II was important for kinase activity (44, 45). Conservation of these viral protein kinases among the Herpesviridae family and their homology to cellular serine kinases indicate indispensability for herpesvirus survival. A number of studies using kinase-null viral mutants demonstrated the importance of the kinase for regulating viral gene expression, replication, or tissue tropism. HSV UL13 kinase mediates post-translational processing and influences expression of several viral genes (46). Human cytomegalovirus UL97 kinase-null mutants show replication deficiency in tissue culture, and the UL97 protein kinase is important for phosphorylation of multiple cellular targets (13, 47, 48). VZV ORF47 null mutants show replication deficiency and the inability to phosphorylate several viral proteins (15, 49, 50). In addition to phosphorylating viral targets, cellular targets including casein kinase II β, EF-1α, p60, and RNA polymerase II serve as substrates for herpesvirus protein kinases (5, 42, 51–53). Demonstrating conserved function between herpesvirus protein kinases, chimeric viruses expressing substituted protein kinases from other herpesviruses are partially able to compensate for lost function (54). In addition, these viral protein kinases were found in purified virus particles, suggesting a role in the assembly of virions or in virion entry into cells (10, 12). Activation of the mitogen-activated stress kinase pathway is important for enhancing herpesvirus replication. Infection with HSV-1 resulted in the activation of JNK, c-Jun, p38, and AP1; enhancement of viral replication (19, 20); and phosho-
rylation of the transcription factor Sp1 (21). During VZV infection, activation of c-Jun, Fos, ATF-2, and AP1 was important for regulating viral genes (23), whereas activation of stress-activated MAP kinases up-regulated transgenes in cytomegalovirus infection (22). These observations on other herpesviruses have implications for our findings on the KSHV ORF36 kinase. Accordingly ORF36 might enhance viral replication by activating c-Jun N-terminal kinase and hence modulating cellular transcription by activation of transcription regulated by c-Jun. KSHV and EBV also encode proteins that are capable of activating the mitogen-activated stress kinase pathway (24–26). Functional studies to demonstrate the significance of these findings will provide clues to the relevance of the mitogen-activated stress kinase pathway in herpesvirus survival and transcription. Such studies could be based on the analysis of kinase-null mutants of the virus, dominant negative JNK pathway mutants, and specific inhibitors for the JNK pathway.

Another possible role for ORF36 phosphorylation of JNK may be the activation of transcription factors encoded by KSHV. Marek disease virus, also a herpesvirus, encodes MEQ, which mimics c-Jun. The MEQ protein shares extensive homology with the Jun/Fos family of transcription factors within the basic region-leucine zipper (bZIP) domain. In addition, MEQ dimerizes with itself and other cellular transcription factors and can functionally substitute for c-Jun (55). KSHV also encodes a gene with a basic region-leucine zipper domain; this protein has been designated K-bZIP and shows homology with BZLF1, an EBV protein essential for viral reactivation and replication (56, 57). These observations suggest that ORF36 plays a role in the activation of KSHV transcription by activating viral homologs of cellular c-Jun. Consequently KSHV ORF36 (a viral late protein) might target an immediate early viral protein, such as K-bZIP. This proposed model is supported by the finding that herpesvirus protein kinases are found in purified virions (10, 12). In fact, using in vitro kinase assays, ORF36 was shown to phosphorylate K-bZIP. Future studies to define the role of ORF36 in KSHV replication and

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**FIG. 5.** Phosphorylation state and association of KSHV ORF36 with MKK4, MKK7, and JNK. A, transfected 293T cells were lysed and immunoprecipitated with FLAG antibody. Western blot analysis of the immunoprecipitated FLAG-tagged ORF36 protein revealed association with MKK4, MKK7, and JNK (lanes 2 and 3). B, transfected 293T cells were immunoprecipitated with either MKK4, MKK7, or JNK antibody. Western blot analysis using FLAG antibody detected FLAG-tagged ORF36 (lanes 2, 3, 5, 6, 8, and 9). The phosphorylation state of KSHV ORF36 was not essential for this association (lanes 3, 6, and 9). IP, immunoprecipitation.

**FIG. 6.** Phosphorylation of JNK and c-Jun during KSHV reactivation and their significance in lytic viral gene expression. KSHV reactivation and viral lytic gene expression were induced in the TREx BCBL1-Rta cell line using doxycycline (1 μg/ml) for 24 h (lanes 3, 4, and 5). In addition to doxycycline treatment, cells were treated with 50 nM of JNKi II (SP600125) (lane 4) or 20 μg/ml JNK inhibitor II negative (JNKi neg) control (lane 5). Cells were not induced (lane 1) or were treated with anisomycin (Aniso) (lane 2). Doxycycline-induced expression of Rta was detected in lanes 3, 4, and 5. Expression of Rta, and lytic replication phosphorylated JNK and c-Jun (lane 3) in these cells. Expression of Rta in the presence or absence of JNK II resulted in the expression of K-bZIP (lanes 3, 4, and 5). Inhibition of K8.1 expression in the presence of Rta and K-bZIP expression was observed in the presence of JNKi II (lane 4). Equal loading of cell lysates was confirmed using anti-tubulin antibody. Tet, tetracycline; p-, phospho-.

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2 M. S. Hamza, R. A. Reyes, Y. Izumiya, R. Wisdom, H.-J. Kung, and P. A. Luciw, unpublished results.
transcription will provide clues into the mechanism by which herpesviruses modulate host cell signaling pathways and maintain their survival within the infected host.

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