Kaposi’s sarcoma-associated herpesvirus (human herpesvirus-8) open reading frame 36 protein is a serine protein kinase

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Kaposi’s sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus that is implicated in the pathogenesis of Kaposi’s sarcoma. The nucleotide sequence of the KSHV open reading frame (ORF) 36 predicts a polypeptide with significant sequence homology to known protein kinases. In this paper, we show that KSHV ORF36 mRNA is expressed during lytic growth and that ORF36 protein is localized in the nucleus. To determine whether the KSHV ORF36 protein is a protein kinase, we expressed it as a glutathione S-transferase (GST) fusion protein (GST–ORF36). Affinity-purified preparations of the GST–ORF36 fusion protein revealed that the protein is autophosphorylated. Mutation of lysine-108 to glutamine dramatically decreased the protein kinase activity of the purified protein, supporting the hypothesis that the protein kinase activity is inherent to the ORF36 protein. Phosphoamino acid analysis showed that the KSHV ORF36 fusion protein is phosphorylated on a serine residue, implying that KSHV ORF36 encodes a serine protein kinase.

Members of the herpesvirus family are divided into three subfamilies, the Alpha-, Beta- and Gammaherpesvirinae, according to their biological properties and genomic structures (Roizman, 1990). Kaposi’s sarcoma-associated herpesvirus (KSHV; also designated human herpesvirus-8) is a gammaherpesvirus that is implicated as a major agent in the formation of Kaposi’s sarcoma (KS), primary effusion lymphomas (PEL) and a large fraction of the cases of Castleman’s disease (Arvanitakis et al., 1996; Boshoff et al., 1995; Cesarm et al., 1995; Chang et al., 1994; Mesri et al., 1996; Soulier et al., 1995). The genomic structure of KSHV is similar to that of herpesvirus saimiri (HVS), with a single contiguous 140-5 kb long unique region containing all identified coding regions, flanked by high G + C terminal repeat units (Russo et al., 1996). DNA sequence analysis of the KSHV genome reveals a number of cellular gene homologues that could contribute to the pathogenesis of KSHV (Neipel et al., 1997b). These include a Bcl-2 homologue (Sarid et al., 1997), a viral cyclin (Li et al., 1997), a viral FLICE inhibitory protein (FLIP), a virus-encoded G protein-coupled receptor (Arvanitakis et al., 1997; Cesarm et al., 1996), a viral interferon regulatory factor (Li et al., 1998), an interleukin-6 homologue (Moore et al., 1996; Neipel et al., 1997a) and a viral macrophage inhibitory protein (MIP)-1 (Moore et al., 1996).

All herpesviruses display two modes of replication: lytic and latent. During lytic replication, the host cells are destroyed and viral progeny particles are released. In contrast, during latent replication, viral genomes persist but show restricted gene expression, and viral progeny is not released (Roizman, 1990). KSHV persists as a circular episome during latency and is able to reactivate and replicate in response to outside stimuli in cultured B cells. Only a small number of viral genes are transcribed during KSHV latency (Zhong et al., 1996).

Many herpesviruses encode putative viral protein kinases (Smith & Smith, 1989). The protein kinases of herpesviruses were first identified in herpes simplex virus (HSV), varicella-zoster virus (VZV) and Epstein–Barr Virus (EBV). HSV US3 and VZV open reading frame (ORF) 66 encode related protein kinases that are specific to the alphaherpesvirus subfamily. Gammaherpesviruses also have other putative protein kinases. EBV BGLF4, HVS ORF36 and KSHV ORF36 show protein kinase domains in their amino acid sequences, although their kinase activity has not yet been verified. There is mounting evidence that protein phosphorylation is essential for virus replication (Lin & Broyles, 1994; Lin et al., 1992). In order to determine whether KSHV ORF36 is a protein kinase, as suggested by Russo et al. (1996), we expressed ORF36 as a fusion protein with glutathione S-transferase (GST) in mammalian cells. The purified GST–ORF36 fusion protein was shown to display protein kinase activity, and one of its serine residues was preferentially phosphorylated. We also found that the KSHV protein kinase is localized in the nucleus.

Comparative analysis of the deduced amino acid sequence revealed that ORF36 contained a catalytic domain found in serine protein kinases. The domain spans 270 amino acids, from amino acid positions 80 to 350 in KSHV ORF36 (Fig. 1). This domain can be divided into 11 subdomains and contains all of the amino acid residues that are normally conserved in serine/threonine protein kinases (Louise et al., 1998). Because

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KSHV ORF36 is known to relate to ORF3 of HVS and BGLF4 of EBV, we aligned the encoded amino acid sequences, obtained from the protein sequence database. These comparisons revealed that the catalytic domain of KSHV ORF36 protein is homologous to those of HVS ORF36 and EBV BGLF4. Conserved subdomain I of KSHV ORF36 contains the glycine loop (GXGXXG), which is important for orienting the phosphates of the ATP substrate. Subdomain VIb has the catalytic loop, which contains a conserved aspartic acid at position 201. Subdomain VII has the conserved sequence DXG, which includes an aspartic acid that is highly conserved in all protein kinases and is important in the binding of an ATP-chelating metal (Louise et al., 1998).

To determine the stage of the virus life-cycle in which KSHV ORF36 protein is expressed, BCBL-1 cells were treated with tetradecanoxyphorol acetate (TPA) in combination with the protein synthesis inhibitor cycloheximide (CH), or the herpesvirus DNA polymerase inhibitor phosphonoacetic acid (PAA). BCBL-1 cells were treated with TPA as previously described (Renne et al., 1996). Total RNA (10 µg) was loaded in each lane and transferred to a nylon membrane after electrophoresis. Upper panel: the filter was hybridized with a KSHV ORF36-specific probe. Lower panel: RNA was assayed by ethidium bromide staining.

To define the subcellular location of KSHV ORF36 protein, we transiently transfected 293 cells with either a green fluorescent protein (GFP)-expression plasmid (pEGFP) or an expression plasmid (pEGFP–ORF36) that contained a GFP-tagged version of KSHV ORF36. Forty-eight hours after transfection, the cells were fixed with 3% formaldehyde in PBS, pH 7-4. Subsequent fluorescent microscopy analysis demonstrated that the GFP–ORF36 fusion protein was localized exclusively in the nucleus (Fig. 2b, right panel).

To decipher the in vivo functions of the KSHV ORF36 protein, we cloned KSHV ORF36 DNA into pEBG, a GST-containing eukaryotic expression plasmid driven by the elongation factor (EF)-1α promoter, to express an ORF36...
ORF36 fusion protein (Lin et al., 1992). To assess the biochemical functions of KSHV ORF36, we performed in vitro kinase assays using GST affinity-purified ORF36 fusion protein (Lin et al., 1992). pEBG, GST–KSHV ORF36 and GST–KSHV ORF36 (K108Q) plasmids were transiently transfected into COS-1 cells by the DEAE-dextran transfection method (Luthman & Magnusson, 1983). COS-1 cell lysates expressing GST, GST–ORF 36 fusion protein and the kinase-dead ORF36 mutant were incubated with glutathione–agarose (35 µl) (Amersham). Before performing the kinase assay, the glutathione-bound complexes were washed once with kinase wash buffer (KWB) [20 mM HEPES (pH 7.5), 5 mM MnCl$_2$, 10 mM β-mercaptoethanol] and resuspended in 25 µl of the same solution supplemented with 10 µCi [$\gamma$-$\text{32P}$]ATP. The phosphorylated proteins were then separated by 7.5% SDS–PAGE, and the gel was subjected to autoradiography. A single prominent band at the expected size of 76 kDa was observed only in the cell lysate transfected with GST–KSHV ORF36 (the wild-type KSHV kinase) (Fig. 3a, left panel, lane 2). In contrast, phosphorylation of the GST–KSHV ORF36 (K108Q) mutant was dramatically decreased compared to that of wild-type KSHV ORF36 (Fig. 3a, left panel, lane 3). These results indicated that KSHV ORF36 protein displayed an intrinsic protein kinase activity and was autophosphorylated. Expression of the recombinant ORF36 protein and ORF36 (K108Q) mutant was verified by immunoblotting using a monoclonal antibody to GST (Fig. 3a, left panel).

Because protein kinases generally require an ATP–divalent cation complex as the phosphate donor (Sun & Budde, 1997), they have an absolute requirement for divalent metal cations. We therefore determined the divalent cation preference of KSHV ORF36 by measuring its autophosphorylation kinase activity in the presence of various concentrations of Mg$^{2+}$ or Mn$^{2+}$ (Fig. 3a, right panel). Autophosphorylation activity was

![Fig. 3. Kinase activity of the KSHV ORF36 protein and phosphoamino acid analysis of GST–ORF36 fusion protein. (a) Kinase activity. Left panel: expression plasmids for GST, wild-type GST–ORF36 fusion protein and its kinase-dead mutant version were transiently transfected into COS-1 cells. Cell lysates were purified on glutathione–Sepharose and assayed for in vitro kinase activity. The upper panel shows the autophosphorylation of KSHV ORF36 fusion protein (arrow). Relative molecular size standards (kDa) are indicated on the left. The lower panel shows the expression of the KSHV ORF36 fusion protein and its kinase-dead mutant (arrow) by immunoblotting with an anti-GST monoclonal antibody. Right panel: effect of divalent cations on the kinase activity of KSHV ORF36 protein. Protein kinase activity was assayed in the presence of various concentrations of MgCl$_2$ and MnCl$_2$. Clarified lysates were pooled and divided into equal aliquots prior to isolation on glutathione–agarose to ensure that all in vitro reactions contained equal amounts of protein. The activity was quantified (graph) by phosphoimage analysis (see panel below graph). The PSL unit was used for the calibration of protein phosphorylation level. PSL calculates the intensity of the selected region. The results are representative of three independent experiments with similar results. (b) Phosphoamino acid analysis of GST–ORF36 fusion protein (lane 1) and GST–ORF36 (K108Q) mutant (lane 2). The HCl digests of the phosphorylated GST–ORF36 fusion proteins and the standards phosphoserine, phosphothreonine and phosphotyrosine were subjected to electrophoresis on parallel lanes on a TLC plate that was subsequently treated with ninhydrin and analysed with a Fujix BAS-1500 (Fuj). Migration positions of the various species are indicated with arrows.](image-url)
We expressed the KSHV ORF36 gene product as a GST fusion regulatory protein. It has also been reported that HSV UL13 phosphorylates the herpesviruses, HSV UL13, VZV ORF47 and EBV BGLF4 homology with other known herpesvirus protein kinases. In these studies of related viral kinases, we suggest that KSHV ORF36 may function in the virus growth-cycle, infection in host cells or replication of the virus. However, further study is required to decipher the true physiological function(s) of the KSHV ORF36 protein.

References


Leopardi, R., Ward, P. L., Ogle, W. O. & Roizman, B. (1997). Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and growth in human T cells and in skin (Mofat et al., 1998). From this series of experiments, we found that the KSHV ORF36 protein kinase preferred Mn2+ to Mg2+ for maximal activity, with an optimal concentration of 5 mM MnCl2.

We next sought to determine which amino acid of the KSHV ORF36 protein was autophosphorylated. KSHV ORF36 protein was autophosphorylated with γ-[32P]ATP and subjected to SDS-PAGE. The phosphorylated polypeptide was eluted from the gel. After elution, the protein-containing solution was mixed with 25 μg carrier BSA. The proteins were precipitated with 100% trichloroacetic acid followed by incubation at 4 °C for 16 h. The protein pellet was dissolved in 50 μl of constant-boiling HCl, boiled at 100 °C for 2 h, and dried. The phosphaomic acids were resolved by electrophoresis on thin-layer cellulose (TLC) plates in pH 1-9 buffer. The migration positions of the phosphoserine, phosphothreonine and phosphotyrosine standards were identified by ninhydrin staining. Our results clearly demonstrated that mainly serine was autophosphorylated by the KSHV ORF36 kinase (Fig. 3b). Phosphorylation of threonine and tyrosine residues could not be detected by this method. The KSHV ORF36 (K108Q) mutant was also tested for its amino acid autophosphorylation specificity. Although the kinase activity of the mutant protein was very low, the pattern of phosphoamino acid specificity was similar to that of wild-type KSHV ORF36. We conclude from these results that the KSHV ORF36 kinase is a serine protein kinase.

In summary, it was predicted from the DNA sequence that the KSHV ORF36 encoded a protein kinase (Russo et al., 1996). We expressed the KSHV ORF36 gene product as a GST fusion protein and demonstrated that it did indeed have a protein kinase activity. Phosphoamic acid analysis of the autophosphorylated KSHV ORF36 fusion protein demonstrated that the kinase activity is specific for serine residues. The in vivo function of and physiologically significant protein substrates for KSHV ORF36 remain a matter of conjecture, but we can hypothesize with respect to its function on the basis of its homology with other known herpesvirus protein kinases. Related protein kinases have been identified in genes in three herpesviruses, HSV UL13, VZV ORF47 and EBV BGLF4 (Smith & Smith, 1989), and we showed that KSHV ORF36 has homology with EBV BGLF4. The HSV UL13 protein kinase has been purified and its activity verified (Daikoku et al., 1997). It has also been reported that HSV UL13 phosphorylates the regulatory protein α22 (Purves et al., 1993), which in its phosphorylated form transactivates the alpha 22 promoter (Leopardi et al., 1997). These reports suggest that the function of UL13 is to provide optimal expression of selected viral proteins (Carter & Roizman, 1996). In addition, it was reported recently that elongation factor (EF)-1α is hyperphosphorylated by HSV UL13, and EF-1α is the first cellular protein that has been shown to be a target for HSV UL13 (Kawaguchi et al., 1998). Another viral kinase that is related to UL13 is the VZV ORF47 protein. This was shown to be required for virus growth in human T cells and in skin (Mofat et al., 1998).


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