Structure of the Open Reading Frame 49 Protein Encoded by Kaposi's Sarcoma-Associated Herpesvirus

Kelly Hew,^a Saranya Veerappan,^a Daniel Sim,^a Tobias Cornvik,^a Pär Nordlund,^{a,b,c} Sue-Li Dahlroth^a

Division of Structural Biology and Biochemistry, Nanyang Technological University, School of Biological Sciences, Singapore^a; Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden^b; Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore^c

ABSTRACT Herpesviruses alternate between the latent and the lytic life cycle. Switching into the lytic life cycle is important for herpesviral replication and disease pathogenesis. Activation of a transcription factor replication and transcription activator (RTA) has been demonstrated to govern this switch in Kaposi's sarcoma-associated herpesvirus (KSHV). The protein encoded by open reading frame 49 from KSHV (ORF49_{KSHV}) has been shown to upregulate lytic replication in KSHV by enhancing the activities of the RTA. We have solved the crystal structure of the ORF49_{KSHV} protein to a resolution of 2.4 Å. The ORF49_{KSHV} protein has a novel fold consisting of 12 alpha-helices bundled into two pseudodomains. Most notably are distinct charged patches on the protein surface, which are possible protein-protein interaction sites. Homologs of the ORF49_{KSHV} protein in the gammaherpesvirus subfamily have low sequence similarities. Conserved residues are mainly located in the hydrophobic regions, suggesting that they are more likely to play important structural roles than functional ones. Based on the identification and position of three sulfates binding to the positive areas, we performed some initial protein-DNA binding studies by analyzing the thermal stabilization of the protein in the presence of DNA. The ORF49_{KSHV} protein is stabilized in a dose-responsive manner by double-stranded oligonucleotides, suggesting actual DNA interaction and binding. Biolayer interferometry studies also demonstrated that the ORF49_{KSHV} protein binds these oligonucleotides.

IMPORTANCE Kaposi's sarcoma-associated herpesvirus (KSHV) is a tumorigenic gammaherpesvirus that causes multiple cancers and lymphoproliferative diseases. The virus exists mainly in the quiescent latent life cycle, but when it is reactivated into the lytic life cycle, new viruses are produced and disease symptoms usually manifest. Several KSHV proteins play important roles in this reactivation, but their exact roles are still largely unknown. In this study, we report the crystal structure of the open reading frame 49 protein encoded by KSHV (ORF49_{KSHV}). Possible regions for protein interaction that could harbor functional importance were found on the surface of the ORF49_{KSHV} protein. This led to the discovery of novel DNA binding properties of the ORF49_{KSHV} protein. Evolutionary conserved structural elements with the functional homologs of ORF49_{KSHV} were also established with the structure.

KEYWORDS Herpesvirus, Kaposi's sarcoma-associated herpesvirus, KSHV, ORF49, structural biology

Kaposi's sarcoma-associated herpesvirus (KSHV) is a human herpesvirus. Herpesviruses are a group of large double-stranded deoxyribonucleotide (dsDNA) viruses of which KSHV is the most recently identified human herpesvirus. KSHV has been found to be the causative agent of human cancers and lymphoproliferative diseases, including

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Address correspondence to Pär Nordlund, par.nordlund@ki.se, or Sue-Li Dahlroth, sdahlroth@ntu.edu.sg.

Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease (1–3).

All herpesviruses exist and propagate indefinitely in their hosts by having two distinct life cycles—the latent and the lytic life cycles (4). During the latent life cycle, the virus exists as a circular episome tethered to the host cell chromosome. The latent life cycle is characterized by limited expression of herpesvirus proteins, which are usually associated with maintenance of the viral episome and host immune evasion (5). Occasionally, the virus may be reactivated from the latent to the lytic life cycle, and disease symptoms usually surface. Even though the process of reactivation is not well defined, the overall determining factor is a shift in immune response brought about by immune suppression related to factors like organ transplants or AIDS, fever, stress, UV light exposures, and even menstruation (6–12). During the lytic life cycle, the full repertoire of herpesvirus proteins is expressed in a cascaded fashion, starting from the immediate early genes to the early genes and finally the late genes (13). New infectious virus particles are assembled and subsequently released upon host cell lysis (14).

KSHV encodes a protein called replication and transcription activator or RTA. RTA is a transcription factor that has been determined to be the molecular switch responsible for reactivation and is sufficient to drive the expression of all KSHV lytic genes (15–17). Another KSHV-encoded protein, the open reading frame 49 protein (ORF49_{KSHV}), has been shown to upregulate the transcriptional activity of RTA (18). The protein homologs of ORF49_{KSHV} in Epstein-Barr virus (EBV) and murine herpesvirus 68 (MHV-68) display similar effects in the respective viruses (19–22). ORF49_{KSHV} has been demonstrated to enhance lytic replication in KSHV, while the lack of the ORF49_{MHV-68} protein has been shown to attenuate viral growth of MHV-68 (23). It is likely that the complementing effects of ORF49_{KSHV} and its homologs on the lytic switch protein RTA are important for the lytic replication in the gammaherpesvirus subfamily.

To date, there is a lack of structural information on individual herpesviral proteins. To increase the knowledge of the ORF49_{KSHV} protein and its homologs, we sought to characterize the protein by solving the crystal structure. Structural analysis of ORF49_{KSHV} revealed a novel fold with sulfate ions binding to the distinctively charged protein surface. These data prompted DNA binding studies of the ORF49_{KSHV} protein using a thermal stability shift assay (TSSA) and biolayer interferometry (BLI). Both TSSA and BLI were able to demonstrate nonspecific DNA binding by the ORF49_{KSHV} protein with low micromolar affinities. DNA binding may contribute to the function of ORF49_{KSHV} during lytic replication. By comparing the locations and interactions of conserved amino acid residues in all homologs and also analyzing the secondary-structure predictions, it is with confidence that we propose that all ORF49_{KSHV} protein homologs have the same overall fold and structure. It, however, remains to be shown if all homologs display the same DNA binding properties as the KSHV protein.

RESULTS

Crystal structure of the ORF49_{KSHV} protein. The full-length ORF49_{KSHV} protein (amino acids 1 to 301) was cloned, expressed, purified, and crystallized. The structure was determined with the single anomalous dispersion (SAD) method and subsequently refined at a final resolution of 2.4 Å (Table 1). There is one ORF49_{KSHV} molecule in each asymmetric unit, and each molecule comprises amino acids 10 to 300, with the exception of a disordered region from amino acids 135 to 143. Two point mutations (Q140P and Q179E) were identified in the amino acid sequence; both were verified by DNA sequencing and determined to have occurred during cloning.

The ORF49_{KSHV} protein has an elongated structure made up of 12 alpha-helices (Fig. 1a). A structural comparison of the ORF49_{KSHV} protein against protein databases showed that ORF49_{KSHV} adopts a novel fold (24, 25). A long single helix, α 3, spans the entire length of the structure and kinks in the middle, creating a slight “V” shape with a groove on one face of the protein surrounded by two pseudodomains. Pseudodomain 1 consists of the N-terminal part of α 3 (residues 41 to 69) that creates a helical bundle with alpha-helices α 1 and α 2 and α 10 to α 12 (Fig. 1b, top). Pseudodomain 2

TABLE 1 Data collection and refinement statistics

Parameter	Value for native protein (PDB ID 5IPX)
Data collection statistics	
X-ray source	AS MX2
Wavelength (Å)	1.549
Space group	C222 ₁
Unit cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	87.6, 142.5, 71.2
$\alpha = \beta = \gamma$ (°)	90
Resolution range (Å)	28.6–2.4 (2.5–2.4) ^a
<i>I</i> / σ (<i>I</i>)	43.8 (11.0) ^a
Completeness (%)	100 (99.4) ^a
Redundancy	10.2 (10.2) ^a
<i>R</i> _{merge} ^b	4.7 (18.8) ^a
No of total reflections	35,329
No. of unique reflections	17,810
Phasing ^c	
No. of sites	3
Initial FOM	0.27
FOM after DM	0.64
Refinement statistics	
<i>R</i> _{work} ^d (%)	21.0
<i>R</i> _{free} ^e (%)	25.3
No. of protein residues	282
No. of solvent molecules	27
Ligand	3 sulfate ions
RMSD bonds (Å)	0.006
RMSD angles (°)	0.61
Ramachandran plot (%)	
Favored regions	97.0
Allowed regions	3.0
Outliers	0.0
Rotamer outliers	1.0

^aThe values in parentheses are for the highest-resolution shell (2.5 to 2.4 Å).

^b $R_{\text{merge}} = \frac{[\sum_{hkl} \sum_i |I_i(hkl) - \bar{I}(hkl)|]}{[\sum_{hkl} \sum_i \bar{I}(hkl)]} \times 100$, where I_i is the *i*th intensity measurement of reflection *hkl* and $\bar{I}(hkl)$ is the mean intensity measurement of the symmetry-related or replicated reflections of the unique reflection *hkl*.

^cFOM, figure of merit; DM, density modification.

^d $R_{\text{factor}} = \frac{[\sum_{hkl} |F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl)|]}{[\sum_{hkl} F_{\text{obs}}(hkl)]} \times 100$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.

^e*R*_{free} is equivalent to *R*_{factor}, but 5% of the measured reflections have been excluded from refinement and set aside for cross validation.

consists of the C-terminal part of $\alpha 3$ (residues 70 to 89) and helices $\alpha 4$ to $\alpha 9$ (Fig. 1b, bottom). The entire protein is stabilized by coiled-coil interactions, and in both pseudodomains, the majority of the helices are slightly twisted around each other (Fig. 1b). The two point mutations mentioned above are located within a disordered surface loop (Q140P) and the protein surface (Q179E), respectively, and do not seem to disrupt the structural integrity of the protein or to affect the structural analysis of the ORF49_{KSHV} protein.

Three sulfate ions (S1 to S3) were successfully modeled into the positive spherical electron densities on the surface of each ORF49_{KSHV} molecule (Fig. 1a). These sulfate ions are likely to have been contributed from the crystallizing solution. S1 is found near the C-terminal end of the protein and is directly coordinated by His 270 and Arg 273 on $\alpha 11$ (Fig. 1c). S2 is located near $\alpha 6$ and $\alpha 7$ and is coordinated by Arg 160 and Arg 164 (Fig. 1d). S2 also binds to the main chain amide of Gly 157 and the side chain of Ser 162 through a water-mediated interaction involving W19. S3 is located in a patch of histidine residues on $\alpha 12$ and is coordinated by His 293 and His 296 (Fig. 1e).

An electrostatic potential surface map of the protein surface reveals that ORF49_{KSHV} has a clear charge separation. One face of the protein has three characteristically

FIG 1 Structure of the ORF49_{KSHV} protein. (a) The ORF49_{KSHV} protein has an elongated structure that is made up of 12 alpha-helices. The long $\alpha 3$ spans the length of the protein and kinks in the middle, dividing the protein into two distinct pseudodomains. (b) Each side of $\alpha 3$ interacts with a bundle of helices that are slightly twisted around each other. (c to e) Three sulfate ions are found on the ORF49_{KSHV} protein surfaces.

charged patches. On this face, there are two positively charged patches flanking a negatively charged area in the middle (Fig. 2, side view 1). The opposite face of the protein is also highly charged (Fig. 2, side view 2). One end of this face is largely hydrophobic, while the other end is predominantly negative. The groove created by the protein's slight "V" shape is largely hydrophobic (Fig. 2, top view). Conversely, the underside of the groove is highly charged with patches of negatively and positively charged surfaces (Fig. 2, bottom view). Sulfate ions are found on the positively charged surfaces of the protein.

DNA binding studies of the ORF49_{KSHV} protein using a TSSA and BLI. The DNA binding abilities of ORF49_{KSHV} were explored with different oligonucleotides, including the KSHV promoter DNA sequences of viral interleukin-6 (vIL-6), kaposin, ORF57, and polyadenylated nuclear RNA (PAN), as well as a random 28-mer (dsO₂₈) and a random 14-mer (dsO₁₄) oligonucleotide, using a TSSA (26). All oligonucleotides induced increased thermal stability to the melting temperature (T_m) of the ORF49_{KSHV} protein, suggesting DNA binding by the protein. A 100 mM concentration of vIL-6 increased the T_m of the ORF49_{KSHV} protein by 4.97 (± 0.07)°C (Fig. 3a). Similarly, kaposin, ORF57, PAN, dsO₂₈, and dsO₁₄ also increased the T_m of the ORF49_{KSHV} protein by 3.14 (± 0.04)°C, 3.88 (± 0.07)°C, 3.50 (± 0.03)°C, 2.96 (± 0.50)°C, and 2.37 (± 0.18)°C, respectively (Fig. 3a). All six oligonucleotides also induced a dose-dependent increment in the T_m of ORF49_{KSHV} protein (Fig. 3b). There was no detectable thermal stabilization by the oligonucleotides on human isocitrate dehydrogenase (NADP) cytoplasmic protein (IDH1), a non-DNA-binding citric acid cycle protein with a protein isoelectric point similar to that of ORF49_{KSHV} (Fig. 3a) (27).

FIG 2 Electrostatic potential surface map of ORF49_{KSHV} protein. Four different orientations of the protein show that the surface of ORF49_{KSHV} protein is characteristically charged with large areas of positively charged (blue), negatively charged (red), and hydrophobic (white) surfaces. Sulfate ions (S1 to S3), shown as spheres, are found binding to the distinctively charged protein surfaces of ORF49_{KSHV}.

DNA binding kinetics of the ORF49_{KSHV} protein measured using biolayer interferometry (BLI) further verified its DNA binding abilities. The ORF49_{KSHV} protein could bind the promoter regions of vIL-6, kaposin, ORF57, and PAN with a K_d (dissociation constant) of $2.56 (\pm 0.08) \mu\text{M}$, $5.48 (\pm 0.34) \mu\text{M}$, $9.05 (\pm 0.82) \mu\text{M}$, and $2.58 (\pm 0.07) \mu\text{M}$, respectively (Fig. 4). However, the ORF49_{KSHV} protein could also bind dsO₂₈ with a K_d of $5.35 (\pm 0.82) \mu\text{M}$ and dsO₁₄ with a K_d of $5.68 (\pm 0.44) \mu\text{M}$ (Fig. 4).

Crystallization screening of ORF49_{KSHV} with oligonucleotides has been attempted but has so far been unsuccessful.

Sequence conservation in the ORF49 protein family. The KSHV- and MHV-68-encoded ORF49 as well as the EBV-encoded Na protein are positional and functional homologs with limited sequence similarities (Fig. 5a). In an attempt to identify the important potential functional regions, the amino acid sequences of ORF49_{KSHV}, ORF49_{MHV-68}, and Na protein were aligned. Identical and similar amino acids were mapped onto the ORF49_{KSHV} protein structure. Conserved residues are localized in

FIG 3 DNA binding studies of ORF49_{KSHV} using TSSA. (a) The melting temperature (T_m) of ORF49_{KSHV} protein was increased by the KSHV promoter regions of vIL-6, ORF57, PAN, kaposin, and two unrelated oligonucleotides, dsO₂₈ and dsO₁₄, in the TSSA. However, this was not observed with the control protein IDH1, which is a non-DNA binding protein (27). (b) ORF49_{KSHV} with various concentrations of oligonucleotides (vIL-6, ORF57, PAN, kaposin, dsO₂₈, and dsO₁₄) showed a dose-responsive increment in the T_m (ΔT_m) of ORF49_{KSHV}. The stabilization effects on ORF49_{KSHV} appear to be higher with vIL-6, followed by ORF57, PAN, Kaposin dsO₂₈ and dsO₁₄, but the degrees of stabilization are not significantly different.

FIG 4 Bi-layer interferometry sensograms of the binding of different concentrations of ORF49_{KSHV} protein (10 μ M, 5 μ M, 2.5 μ M, 1.25 μ M, 0.63 μ M, and 0 μ M) with the KSHV promoter regions of vIL-6, ORF57, PAN, kaposin, and two unrelated oligonucleotides, dsO₂₈ and dsO₁₄. The perpendicular line in the middle of the sensograms sections the association (left) from the subsequent dissociation (right) of ORF49_{KSHV} protein.

hydrophobic regions along the interacting surfaces of the alpha-helices and most likely play a primarily structural role (Fig. 5b).

DISCUSSION

Reactivation into the lytic life cycle is important for the production of new infectious herpesvirus particles in the host and hence the survival of the virus (14, 28). In KSHV, this is governed by the transcriptional control facilitated by RTA, and studies have demonstrated that ORF49_{KSHV} and its homologs are able to cooperate with RTA to enhance its transcriptional activation (13, 16–18, 20–22). However, the underlying mechanism of this process is still unclear, with limited functional studies and lack of structural information.

The structure of the ORF49_{KSHV} protein was determined to obtain a better understanding of its role in the herpesviral life cycle. The ORF49_{KSHV} protein has an elongated novel fold, and its surface shows a clear charge separation, suggesting several sites for interactions with other proteins and/or charged molecules (Fig. 1 and 2). The structure of ORF49_{KSHV} is not conserved as DNA binding domains of typical transcriptional activators/repressors (24, 25). However, the presence of sulfate ions on its positive surfaces prompted us to investigate the ORF49_{KSHV} protein's potential DNA binding properties with oligonucleotides harboring the KSHV promoter regions upregulated by RTA (Fig. 1 and 2) (26). DNA binding was first investigated with a TSSA, which we have previously used successfully to identify DNA binding for viral proteins (29, 30).

A typical dose-responsive increase in protein stability in the presence of different oligonucleotides suggests that the ORF49_{KSHV} protein is able to bind dsDNA *in vitro* (Fig. 3). Even though the stabilizing effects seem to be slightly stronger with the KSHV promoter DNA sequences than the unrelated oligonucleotides, the difference in T_m is too small to clearly indicate a preference for the promoter sequences (Fig. 3b). BLI also demonstrated similar binding affinities of the ORF49_{KSHV} protein with all the oligonucleotides. Even so, these preliminary results suggest that the ORF49_{KSHV} protein is able to bind DNA of various lengths and sequences.

ORF49_{KSHV} has been shown to increase transcriptional activation in cooperation with RTA (18). Studies with its MHV-68 homolog, ORF49_{MHV-68}, suggested that this cooperation is likely due to interactions between ORF49_{MHV-68} and RTA (21). Since

FIG 5 Comparisons of ORF49_{KSHV} protein homologs. (a) The sequence alignment of ORF49_{KSHV}, ORF49_{MHV-68}, and Na shows limited sequence conservation among the three proteins. (b) The identical (red) and similar (pink) amino acids of ORF49_{KSHV}, ORF49_{MHV-68}, and Na were mapped and shown on the structure of ORF49_{KSHV}. These conserved amino acids are located along the interfaces of the interacting alpha-helices.

ORF49_{KSHV} has been demonstrated to bind DNA *in vitro*, it is tempting to propose that the upregulation of RTA's transcriptional activity by ORF49_{KSHV} might also involve direct DNA binding by ORF49_{KSHV}. Obviously, *in vitro* studies do not necessarily imply that ORF49_{KSHV} will show its DNA binding properties in infected cells.

For future work, it would be interesting to investigate the potential phosphate binding sites and also the DNA binding capacity in the presence of RTA through

targeted mutations. As already mentioned, the sequence conservation between the different homologs is very low, and the residues that are involved in the coordination of the sulfate ions are not conserved. Instead, conserved residues seem to play structural roles rather than functional ones. Even so, the protein structure of ORF49_{KSHV} constitutes the first structure to be solved of any of the ORF49 gammaherpesvirus protein homologs. A comparison of the secondary-structure predictions for the Na protein (data not shown) and the location of conserved residues indicate that Na and ORF49_{MHV-68} have a fold and overall structure similar to those of ORF49_{KSHV}.

In summary, the structure of ORF49_{KSHV} has provided us with insights into the protein, revealing a novel protein fold and potential interaction areas. DNA binding studies of ORF49_{KSHV} also indicate direct binding between the ORF49_{KSHV} protein and dsDNA. The DNA binding characteristics might be a part of the protein's functional role in the lytic replication of KSHV.

MATERIALS AND METHODS

Protein expression. ORF49_{KSHV} was cloned as previously described into pNIC28-Bsa4 with an N-terminal histidine tag to enable affinity purification and immunoblotting (31, 32). The starter culture supplemented with 50 μ g/ml kanamycin and 34 μ g/ml chloramphenicol was inoculated with the ORF49_{KSHV} glycerol stock. The culture was incubated overnight at 37°C and 220 rpm. Ten milliliters of the overnight culture was added to 750 ml of terrific broth (TB) supplemented with 50 μ g/ml kanamycin, 34 μ g/ml chloramphenicol, and approximately 200 μ l of antifoam agent. The cultures were grown in a large-scale expression (LEX) system (Harbinger Biotech) and kept at a constant temperature of 37°C until the optical density at 600 nm (OD₆₀₀) reached 2 to 3. The cultures were cooled to 18°C, and expression was induced with 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) overnight. The cells were harvested by centrifugation (4,500 $\times$ g for 10 min at 15°C) the next day. The pelleted cells were flash frozen with liquid nitrogen, ground, and stored at -20°C . Unless otherwise stated, all reagents were from Sigma-Aldrich.

Protein purification. The ground cell pellets were resuspended in ice-cold lysis buffer (100 mM HEPES [pH 8.0], 500 mM NaCl, 10 mM imidazole [pH 8.0], and 10% glycerol) supplemented with 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), 0.1 mg/ml lysozyme, 1 μ l/ml protease inhibitor cocktail (EDTA free; Nacalai), and 125 U/ml benzonase (Merck Millipore) and sonicated. The lysates were clarified by centrifugation (47,000 $\times$ g for 25 min at 4°C) and filtration through a 1.2- μ m syringe filter. The filtrate was loaded onto a preequilibrated 1-ml HisTrap HP IMAC column (GE Healthcare; 20 mM HEPES [pH 7.5], 500 mM NaCl, 10 mM imidazole, 10% glycerol, 0.5 mM TCEP), and the column was washed with 20 ml of equilibration buffer and 15 ml of wash buffer (20 mM HEPES [pH 7.5], 500 mM NaCl, 25 mM imidazole, 10% glycerol, 0.5 mM TCEP). The protein was eluted with 5 ml elution buffer (20 mM HEPES [pH 7.5], 500 mM NaCl, 500 mM imidazole, 10% glycerol, 0.5 mM TCEP), and the eluate was loaded onto a preequilibrated (20 mM HEPES [pH 7.5], 500 mM NaCl, 10% glycerol, 0.5 mM TCEP) gel filtration column (HiLoad 16/60 Superdex 75; GE Healthcare) directly. The eluted protein samples were collected in 2-ml fractions and analyzed on 4 to 12% NuPAGE Bis-Tris gels. After size exclusion chromatography, the fractions were analyzed on 4 to 12% NuPAGE Bis-Tris gels. The pure ORF49_{KSHV} protein samples were combined, and 2 mM TCEP was added prior to protein concentration. The ORF49_{KSHV} protein was concentrated to 6 mg/ml using a 10-kDa MWCO protein concentrator, before being flash frozen with liquid nitrogen and stored at -80°C .

TSSA. A thermal stability shift assay (TSSA) of purified ORF49_{KSHV} protein with different oligonucleotides was performed using a real-time PCR (RT-PCR) machine (Bio-Rad) with a 96-well PCR plate (Bio-Rad).

Lyophilized oligonucleotides (Integrated DNA Technologies) were solubilized with deionized distilled water. Double-stranded oligonucleotides were prepared by mixing equimolar amounts of the complementary oligonucleotides (for vIL6, 5'-AAATGGGTGGCTAACCTGTCCAAAA and 5'-TTTGGACAGGTTAGC CACCCATT; kaposin, 5'-GGAAATGGGTGGCTAACCCCTACATA and 5'-TATGTAGGGGTAGCCACCCATT TCC; ORF57, 5'-AGTGAACAATAATGTCCACGGC and 5'-GCCGTGGGAACATTATTGTACACT; PAN, 5'-AAACCCGCCCCCTGGTGCTCACTT and 5'-AAAGTGAGCACCAGGGGGCGGGTTT; dsO₂₈, 5'-GCCCCACG CCGGACCCGCGAGACTT and 5'-AAAGTCTCGCGGTCCGGGGCGTGGGGC; dsO₁₄, 5'-TTTAAGATCAGTAC and 5'-GCCCCACGCCCCGACCCGCGAGACTT).

The oligonucleotides were incubated at 95°C for 2 min and cooled at room temperature. Each well contained 0.2 mg/ml of the ORF49_{KSHV} protein, 5 $\times$ SYPRO orange (Life Technologies), and 100 μ M oligonucleotides, marked up to a final volume of 25 μ l with TSSA buffer (20 mM HEPES [pH 7.5] and 300 mM NaCl). Triplicate experiments were done, and TSSA buffer was added in place of the oligonucleotides as blanks. The same experiment was repeated by replacing the ORF49_{KSHV} protein with purified human isocitrate dehydrogenase (NADP) cytoplasmic protein (IDH1) as a control. A dose-responsive TSSA of the ORF49_{KSHV} protein and the oligonucleotides was also performed similarly to that described above, but with different oligonucleotide concentrations: 100 μ M, 50 μ M, 25 μ M, 12.5 μ M, 6.3 μ M, 3.2 μ M, 1.6 μ M, and 0 μ M.

BLI. The DNA-binding kinetics of the ORF49_{KSHV} protein with various oligonucleotides were characterized with an Octet RED96 (ForteBio) biolayer interferometry (BLI) system at 25°C in the assay buffer (10 mM phosphate [pH 7.4], 137 mM NaCl, 2.7 mM KCl, 0.1% bovine serum albumin

[BSA], 0.01% Tween 20). 5'-biotinylated DNA oligonucleotides (IDT) were prepared as described above. The biotinylated oligonucleotides were immobilized onto streptavidin biosensor tips, followed by the measurement of BLI signals upon the association of different concentrations of the ORF49_{KSHV} protein (10 μ M, 5 μ M, 2.5 μ M, 1.25 μ M, 0.63 μ M, and 0 μ M) and the subsequent dissociation of the ORF49_{KSHV} protein into blank assay buffer. The dissociation constant (K_d) of each DNA was obtained by fitting the full titration range of the ORF49 binding data with a 1:1 model of association and dissociation functions (ForteBio).

Crystallization and data collection. The ORF49_{KSHV} protein was crystallized by vapor diffusion in a drop composed of 200 nl of the protein solution (6 mg/ml) and 100 nl of the crystallizing solution containing 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6) (Qiagen), 0.15 M ammonium sulfate (Qiagen), and 15% (wt/vol) polyethylene glycol 4000 (PEG 4000) (Qiagen). The drop was incubated in a 96-well sitting drop Intelli-plate (Art Robbins) at 4°C. The native crystals were soaked in cryoprotectant composed of 0.15 M ammonium sulfate, 0.1 M MES (pH 6), and 32% (wt/vol) PEG 4000 for a minute before being flash frozen in liquid nitrogen.

ORF49_{KSHV} protein also crystallized as a second crystal form in a drop consisting of 150 nl of protein solution (6 mg/ml) and 150 nl of crystallizing solution containing 0.1 M MES (pH 6) (Qiagen), 0.2 M NaCl (Qiagen), and 20% (wt/vol) PEG monomethyl ether (MME) 2000 (Qiagen). The drop was also incubated in a 96-well vapor diffusion sitting drop Intelli-Plate at 4°C. Derivative ORF49_{KSHV} crystals were obtained by soaking these crystals in the heavy-atom solution containing 10 mM gold(III) potassium cyanide (Hampton), 0.1 M MES (pH 6), 0.2 M NaCl, 20% (wt/vol) PEG MME 2000, and 2 mM TCEP for 20 min. The crystals were subsequently dipped into the cryoprotectant, which is made up of 0.1 M MES (pH 6), 0.2 M NaCl, 20% (wt/vol) PEG MME 2000, and 20% (vol/vol) glycerol, before being flash frozen in liquid nitrogen.

Diffraction data were collected at the MX2 Micro Crystallography beam line in the Australian Synchrotron, Australia, using the Blu-Ice data collection software (33). The native and anomalous data sets were collected using the native and derivative ORF49_{KSHV} crystals at wavelengths of 1.0393 Å (gold L-III edge) and 1.5492 Å, respectively. Both data sets were indexed and integrated separately with iMosflm and scaled together with Aimless (34, 35).

Structure determination. The structure of ORF49_{KSHV} was determined using the single-wavelength anomalous dispersion (SAD) method with the anomalous data set. SHELX-C and -D were used for substructure determination, heavy-atom positional refinements, and phase calculations (36). The phases obtained were further improved by density modifications with SHELX-E (36). An initial ORF49_{KSHV} model was built automatically by ARP/wARP (37).

The native data set was used to obtain a higher-resolution native ORF49_{KSHV} structure. The native ORF49_{KSHV} structure was determined with molecular replacement using Phaser and the initial ARP/wARP built model as the search model (37, 38). Inspection of the $F_o - F_c$ electron density maps revealed three large spherical positive densities on the surface of ORF49_{KSHV}. Sulfate ions were modeled manually into the positive electron densities using Coot and refined with phenix.refine (39–41). Further validation with a metal binding site server could not identify any binding of metal ions to the positive electron densities (42).

The final model was obtained after multiple cycles of automated refinements with phenix.refine and manual refinements with Coot (39, 40). The quality of the final model was validated using the comprehensive validation software from the PHENIX suite (43, 44). PDB2PQR and APBS from the APBS plug-in in PyMOL were used to calculate and display the electrostatic potential of the protein solvent-accessible areas of ORF49_{KSHV} (45, 46). All figures of the ORF49_{KSHV} structure were displayed with PyMOL (47).

Accession number(s). The final coordinates of ORF49_{KSHV} were deposited in the PDB under structure ID 5IPX.

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