Identification of Kaposi Sarcoma Herpesvirus (KSHV) vIRF1 Protein as a Novel Interaction Partner of Human Deubiquitinase USP7*

Sara Chavoshi, Olga Egorova, Ira Kay Lacdao, Sahar Farhadi, Yi Sheng, and Vivian Saridakis

From the Department of Biology, York University, Toronto, Ontario M3J 1P3, Canada

Viral interferon regulatory factor 1 (vIRF1), a Kaposi sarcoma herpesvirus protein, destabilizes p53 by inhibiting p53 acetylation and Hdm2 phosphorylation. This leads to increased ubiquitination and degradation of p53 by Hdm2, which cripples the cellular p53-mediated antiviral response. Ubiquitin-specific protease 7 (USP7) deubiquitinates p53 and Hdm2 and regulates their stability. We identified an EGPS consensus sequence in vIRF1, which is identical to that found in Epstein-Barr virus nuclear antigen 1 (EBNA1) that interacts with the N-terminal domain of USP7 (USP7-NTD). GST pulldown assays demonstrated that vIRF1 interacts with USP7-NTD via its EGPS motif. NMR heteronuclear single quantum correlation (HSQC) analysis revealed chemical perturbations after titration of USP7-NTD with vIRF1 \(^4\)SPGEPSGTG\(^{53}\) peptide. In contrast, these perturbations were reduced with a mutant vIRF1 peptide, \(^4\)SPG-EGPAGT\(^{53}\). Fluorescence polarization analysis indicated that the vIRF1 peptide interacted with USP7-NTD with a \(K_D\) of 2.0 \(\mu\)M. The crystal structure of the USP7-NTD-vIRF1 peptide complex revealed an identical mode of binding as that of the EBNA1 peptide to USP7-NTD. We also showed that USP7 interacts with vIRF1 in U2OS cells. Decreased levels of p53, but not Hdm2 or ataxia telangiectasia-mutated (ATM), were seen after expression of vIRF1, but not with a vIRF1 mutant protein. Our results support a new role for vIRF1 through deregulation of the deubiquitinating enzyme USP7 to inhibit p53-mediated antiviral responses.

Human herpesviruses (HHVs)\(^2\) are double-stranded DNA viruses, classified into \(\alpha\), \(\beta\), or \(\gamma\) subfamilies (1). To establish latency, HHVs suppress the host immune response to evade the immune system (2). In the latent state, herpesviruses express only a small number of proteins essential for suppressing the host immune system (2). HHVs have evolved various mechanisms for host immune evasion including inhibition of cellular senescence and apoptosis as well as promoting cell proliferation (3). Another strategy to evade host immune surveillance is through expression of viral homologues of genes that are the host’s first line of defense against viral infection such as interferons and interferon regulatory factors (IRFs) and therefore sabotage the function and regulation of the cellular proteins (4). Deregulation of cellular proteins involved in growth control by HHVs has led to recognition of some HHVs as underlying agents of cancer (3, 5).

Kaposi sarcoma herpesvirus (KSHV), HHV-8, is the causative agent of Kaposi sarcoma, primary effusion lymphoma, and multicentric Castleman disease, which are especially prevalent in immunocompromised patients (4, 6). The KSHV vIRF1 protein is encoded by ORF K9 and is believed to have been acquired through molecular piracy (7). vIRF1 contains two domains, an N-terminal DNA binding domain (DBD) and a C-terminal IRF interaction domain (see Fig. 1A) (8). The vIRF1 DBD has \(~40\%\) sequence similarity to the DBDs of human IRF3 and IRF7 and contains a helix-turn-helix motif, which is common in IRFs and DNA-binding proteins (8). vIRF1-mediated deregulation of IRF3 and IRF7 leads to disruption of cellular antiviral activity. vIRF1 is a potent inhibitor of the histone acetyltransferase activity of p300. It leads to hypoacetylation of histones and alteration of the chromatin structure, reducing expression of IFNs (9). vIRF1 also directly interacts with p53 and inhibits its acetylation by p300 (10). Furthermore, vIRF1 has an inhibitory effect on ataxia telangiectasia-mutated (ATM) kinase (11). ATM activation leads to the phosphorylation of Ser\(^{15}\) on p53 and Ser\(^{195}\) on Hdm2. These phosphorylations disrupt the Hdm2 ubiquitination of p53, leading to its stabilization (11, 12). Also, phosphorylation of Ser\(^{15}\) is a signal for p300 acetylation of p53, which is important for its stability and activation (13). Thus, inhibition of ATM activity by vIRF1 increases ubiquitination and degradation of p53 by Hdm2 (11).

Human ubiquitin-specific protease 7 (USP7), also known as herpes-associated ubiquitin-specific protease (HAUSP), is a deubiquitinating enzyme originally identified as an interacting protein with HSV-1 immediate early protein, ICP0 (14). USP7 harbors a TRAF-like domain in its N terminus (USP7-NTD), a central catalytic domain, and five ubiquitin-like folds in its C terminus (USP7-CTD) (Fig. 1A) (15–17). The USP7-NTD recognizes and binds interacting proteins containing a...
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(P/A/E)XXS motif (15, 18–20). USP7-NTD harbors a 164-DWGF167 motif in its binding pocket where the Asp and Trp residues are essential for protein interaction (18).

The p53 tumor suppressor protein and its negative regulator, the E3 ligase, Hdm2, are substrates of USP7 and interact with the 164-DWGF167 motif of USP7-NTD through their (P/A)XXS motifs (18, 20, 21). Because Hdm2 has a higher affinity for USP7 than p53, reduction in cellular levels of USP7 leads to instability of p53, whereas its removal results in destabilization of Hdm2, which in turn stabilizes p53 (22, 23). USP7 also regulates the stability of HdmX, a homologue of Hdm2 (22). USP7 is an essential component of the p53-Hdm2-HdmX pathway that maintains balance in the cellular levels of these proteins (24).

We identified a 47EGPS50 consensus sequence in vIRF1 that is identical to a motif reported in EBNA1 responsible for mediating its interaction with USP7-NTD. This led us to investigate whether vIRF1 interacts with USP7. We characterized the interaction between USP7-NTD and KSHV vIRF1 using GST pulldown and fluorescence polarization assays, and further mapped the binding interface by two-dimensional NMR HSQC. We also determined the crystal structure of a vIRF1 peptide with USP7-NTD. We confirmed that these two proteins interact in vivo and determined that expression of wild-type vIRF1 but not the 47EGPS50 deletion mutant, ΔvIRF1, decreased cellular levels of p53, but not Hdm2 or ATM.

Experimental Procedures

**Protein Expression**—N-terminal hexahistidine-tagged WT and DI64A/W165A mutant USP7-NTD were expressed from the pET15b vector as described previously (18). His-tagged USP7-CTD was expressed from p15TV-L vector (25). Full-length His-USP7 in pFastBac was expressed in Spodoptera frugiperda (Sp9) cells as described previously (26). Full-length vIRF1 was synthesized by GenScript.

**Peptide Synthesis**—Wild-type (44SPGEPSGTG53) and mutant (44SPGEQPAGTG53) vIRF1 peptides were synthesized by CanPeptide Inc. (Montreal, Canada).

**Fluorescence Polarization Binding Assay**—Both wild-type and mutant USP7-NTD were further purified by size-exclusion chromatography using a HiLoad 16/60 Superdex 200 (GE Healthcare) on an ÄKTApurifier 10 UPC system (GE Healthcare) in 150 mM NaCl and 50 mM Tris, pH 8.0. FITC-labeled WT (FITC-Acp-44SPGEPSGTG53-NH2) and mutant (FITC-Acp-44SPGEQPAGTG53-NH2) vIRF1 peptides were initially dissolved in dimethyl sulfoxide to a final concentration of 10 mM. A 400 nM working stock of each peptide was prepared in assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.01% Triton X-100). 500 μM of each protein was serially diluted and incubated with 40 nM of each peptide. 10 μl of the above mixtures were transferred into a 384-well plate (Corning), and fluorescence polarization was measured on a Synergy H4 microplate reader (BioTek) with λex = 485 nm and λem = 520 nm. Polarization values were analyzed by GraphPad Prism 5.0 using a one-site binding model to obtain the equilibrium dissociation constant (Kd). Data were calculated based on four individual experiments, and the standard deviation was calculated.

**NMR Spectroscopy**—NMR spectra were acquired at 25 °C on aBruker 700-MHz NMR spectrometer equipped with a triple resonance cryoprobe. Interaction of USP7-NTD with WT (44SPGEPSGTG53) and mutant (44SPGEQPAGTG53) vIRF1 peptides was monitored by analyzing 1H-15N HSQC spectra. Briefly, 15N-labeled USP7 was incubated with thrombin to cleave the hexahistidine fusion tag followed by dialysis into NMR buffer (200 mM NaCl, 25 mM sodium phosphate, pH 7.0, and 10 mM DTT). Up to 0.4 mM unlabeled wild type or mutant vIRF1 peptide was titrated in 0.2 mM 15N-labeled USP7 (containing 10% D2O) up to 2:1 peptide:USP7 molar ratio. Spectra were processed with TopSpin 3.2 and analyzed with the SPARKY program (27, 28).

**GST Pulldown Assay**—Cells expressing GST-tagged fusion proteins were lysed using sonication in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM protease inhibitor cocktail (1 mM benzamidine and 0.5 mM PMSF) and 1 mM protease inhibitor tablet (Roche Applied Science Complete ULTRA Tablets). Lysate was cleared and allowed to interact with glutathione-Sepharose beads (GE Healthcare) for 1 h. The beads were washed extensively with 50 mM Tris, pH 7.5, and 150 mM NaCl. GST-tagged WT and deletion mutant vIRF11–90 were kept bound to the glutathione-Sepharose beads. Prior to the GST pulldown assays, USP7 proteins were dialyzed against 100 mM NaCl, 50 mM Tris, pH 8.0, 5% glycerol, 5 mM β-mercaptoethanol and 1 mM protease inhibitor tablet. 15 μl of GST-tagged wild type and deletion mutant vIRF11–90 bound to glutathione-Sepharose resin were incubated with 5 nmol of purified full-length USP7, wild-type USP7-NTD, mutant USP7-NTD, or USP7-CTD for 2 h at 4 °C. 5 nmol of GST alone bound to glutathione-Sepharose beads were used as a negative control. The mixtures were then transferred to micro-columns and washed extensively with assay buffer. The bound proteins were eluted with 20 mM reduced glutathione and detected by Coomassie Blue staining following SDS-PAGE.

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Crystalization—Prior to setting up crystal trials, purified USP7-NTD was incubated with thrombin to remove the hexahistidine N-terminal fusion tag. USP7-NTD was further purified by size-exclusion chromatography using a HiLoad 26/60 Superdex 75 (GE Healthcare) on an AKTApurifier 10 UPC (GE Healthcare) in 500 mM NaCl and 20 mM Hepes, pH 7.5. USP7-NTD (100 mg/ml) was co-crystallized with at least 5-fold molar excess of vIRF1 (44SPGEGPSGTG53) peptide at 4 °C using the hanging-drop vapor diffusion method. Rod-shaped crystals appeared after 4 days following one round of micro-seeding using USP7-NTD-UbE2E1 peptide crystal seeds in 30% PEG 4000, 0.1 M Tris, pH 8.5, and 0.2 M LiSO₄ (29).

X-ray Data Collection and Structure Determination—X-ray data were collected at the Advanced Photon Source (APS). Diffraction data were integrated and scaled using the autoPROC software (30). The structure was determined by molecular replacement employing USP7-NTD (Protein Data Bank (PDB) ID 1YY6, without the EBNA1 peptide) as search model using CNS 1.3 (31). The electron density was visualized and the vIRF1 peptide model was built using Coot (32). CNS was used for refinement and water picking at 1.5 Å resolution. The data collection and refinement statistics are shown in Table 1. Fig. 3 was prepared using PyMOL (33).

Structure Deposition—Coordinates and structure factors for the USP7-NTD-vIRF1 peptide complex have been deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) under PDB ID 4YSI.

Cell Culture and Antibodies—Human osteosarcoma U2OS cells were grown in McCoy’s medium supplemented with 10% FBS and 1 mg/ml penicillin-streptomycin. The antibodies were rabbit polyclonal against USP7 (Bethyl Laboratories, A300-033A), mouse monoclonal against USP7 (Millipore, 05-1946), mouse monoclonal against Myc (Millipore, 05-724), rabbit polyclonal against FLAG tag (Bethyl Laboratories, A190-102A), mouse monoclonal against FLAG tag (Sigma, F3165), mouse monoclonal against p53 (Santa Cruz, sc-126), rabbit polyclonal against phospho-p53 (Ser15) (Cell Signaling, 9284), rabbit monoclonal against ATM (Cell Signaling, 2873), rabbit monoclonal against phospho-ATM (Ser1981) (Cell Signaling, 5883), mouse monoclonal against MDM2 (Santa Cruz, sc-965), rabbit polyclonal against phospho-MDM2 (Ser166) (Cell Signaling, 3521), and mouse monoclonal against GAPDH (Santa Cruz, sc-47724). To detect proteins of interest, HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-035-166) and anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 111-035-003) antibodies were used.

Co-immunoprecipitation—Cells were transfected with pCMV/N-Myc USP7 and pcDNA3.1/FLAG vIRF1 (wild type or EGPS deletion mutant) vectors (total of 10 μg of DNA per 15-cm tissue culture plate) using the Polyjet transfection reagent according to the manufacturer’s protocol (SignaGen Laboratories). Cells were harvested 48 h after transfection and lysed in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 20% glycerol, 1× protease inhibitor cocktail (Roche Applied Science)) followed by 2 s of sonication at 10% amplitude. Cell lysates were incubated with either rabbit polyclonal anti-USP7 or anti-FLAG primary antibodies overnight at 4 °C followed by the addition of pre-cleared protein A/G PLUS-Agarose beads (Santa Cruz, sc-2003) for 1 h. Immunoprecipitates were washed with radioimmunoprecipitation buffer five times and then boiled in SDS sample buffer for 5 min at 95 °C. Samples were resolved on 10% SDS-polyacrylamide gels and immunoblotted using the antibodies described above. The co-immunoprecipitation experiment for endogenous USP7 was carried out using the same protocol.

Immunoblotting—Cells were transfected with pcDNA3.1/FLAG empty vector and pcDNA3.1/FLAG vIRF1 (wild type or EGPS deletion mutant) vectors (1 μg of DNA per 10-cm tissue culture plate) using the Polyjet transfection reagent according to the manufacturer’s protocol (SignaGen Laboratories). Cells were harvested 24 h after transfection and lysed in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1× protease inhibitor cocktail (Roche Applied Science) and 1× phosphatase inhibitor cocktail (Cell Signaling)) followed by 2 s of sonication at 20% amplitude. Supernatants were boiled in SDS sample buffer for 5 min at 95 °C and resolved on SDS-polyacrylamide gels. Immunoblotting was performed using antibodies described above. Bands were quantified and normalized using ImageJ.

Results

Identification of an EGPS Sequence in KSHV vIRF1—It is well established that many USP7 substrates including Hdm2, p53, and HdmX contain (P/A)XXS motifs for interaction with USP7-NTD through the highly conserved 164DWGF167 motif with dissociation constant (Kd) values ranging from 10 to 45 μM (18–20). KSHV proteins including vIRF4 and LANA interact with the USP7-NTD 164DWGF167 motif through (P/A)XXS sequences (34, 35). EBV EBNA1 also interacts with USP7-NTD, although with an unusual motif, EGPS, instead of (P/A)XXS (15). A recent study revealed that ORF45, a KSHV immediate

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TABLE 1

| X-ray data collection and refinement parameters | USP7-NTD-vIRF1 peptide |
|-----------------------------------------------|------------------------|
| **X-ray data**                                 |                        |
| Space group                                   | P₄₁               |
| Resolution (Å)                                | 50.0–1.02            |
| Unit cell axes (Å³)                            | 70.0 × 70.0 × 45.4   |
| Molecules/AU²                                  | 1                    |
| No. of total observations                     | 684,942              |
| No. of unique reflections                     | 111,563              |
| Intensity (I/σ(I))                            | 20.6 (2.0)           |
| Completeness (%)                              | 99.9 (98.2)          |
| Multiplicity                                  | 6.1 (5.5)            |
| Rfree (%)                                     | 0.039 (0.776)        |

**Refinement**

- Rwork = 0.171
- Rsym = 0.176
- No. of protein atoms                        1186
- No. of water molecules                      268
- r.m.s.d. bonds (Å)                          0.008
- r.m.s.d. angles (°)                         1.29
- r.m.s.d. didecrease (°)                     25.2
- r.m.s.d. improper (°)                       0.86
- Thermal factors (Å²)                        13.5
- Ramachandran plot                           Most Favorable 0.95
- Additionall allowed                         0.05

a AU, asymmetric unit.

b Rfree = Σ |I(I)− (<I(I))/2I| where I(1) is the observed intensity and (,) is the average intensity from multiple observations of symmetry-related reflections.
early protein, also interacts with USP7-NTD through an EGPS sequence (36). We utilized ScanProsite to search for viral proteins containing the DPPEGPST sequence of EBNA1 and identified 44SPGEGPSG51 from KSHV vIRF1 (37). Alignment of the USP7 binding motif of vIRF1 with the previously identified USP7 binding motifs of EBV EBNA1 and KSHV ORF45. Asterisks represent conserved residues, and the period represents semi-conserved substitution.

C GST pulldown assay with GST-vIRF11–90, GST-ΔvIRF11–90 (deletion of 44PGEGPS50), or GST alone as negative control. Full-length USP7, USP7-NTD, USP7-CTD, and USP7-NTD-DW were used as prey. L load; E elution.

**FIGURE 1.** vIRF1 interacts with USP7-NTD in vitro. A schematic representation of USP7 and vIRF1 domain organization. USP7 contains an N-terminal substrate binding TRAF domain, a catalytic domain, and a C-terminal domain that is composed of five ubiquitin-like (Ub) sub-domains. vIRF1 contains a DBD and an interferon association domain (IAD). The arrow indicates the location of identified EGPS residues in vIRF1 in an unstructured region close to the DBD. B, alignment of the USP7 binding motif of vIRF1 with the previously identified USP7 binding motifs of EBV EBNA1 and KSHV ORF45. Asterisks represent conserved residues, and the period represents semi-conserved substitution. C GST pulldown assay with GST-vIRF11–90, GST-ΔvIRF11–90 (deletion of 44PGEGPS50), or GST alone as negative control. Full-length USP7, USP7-NTD, USP7-CTD, and USP7-NTD-DW were used as prey. L load; E elution.

**USP7-NTD and vIRF1 Interact in Vitro**—To test the binary interaction between vIRF1 and USP7, a series of GST pulldown assays were performed using GST-tagged vIRF11–90. GST-vIRF11–90 interacted with both full-length USP7 and USP7-NTD but not USP7-CTD, confirming that vIRF1 binding is mediated through USP7-NTD (Fig. 1C). Neither full-length USP7 nor USP7-NTD was retained by GST alone, indicating that the interaction with GST-vIRF11–90 was specific. Deletion of residues 45PGEGPS50 (GST-ΔvIRF11–90) abolished its interaction with both full-length USP7 and USP7-NTD, confirming that vIRF1 interacts with USP7-NTD through its EGPS sequence, which is similar to the USP7 binding motif found in EBNA1 (Fig. 1C). We also performed GST pulldowns using USP7-NTD-DW, a double mutant containing alanine mutations at residues Asp164 and Trp165 within the 164DWGF167 motif. As expected, USP7-NTD-DW did not interact with vIRF1 (Fig. 1C). These pulldown assays demonstrated that USP7-NTD interacts specifically with vIRF1 in vitro.

**Interaction between USP7-NTD and vIRF1—**Fluorescence polarization was used to determine the dissociation constant between USP7-NTD and vIRF1 peptides. FITC-labeled WT (44SPGEPSPGTG53) and mutant (44SPGEPAGTG53) vIRF1 peptides containing the potential USP7 interaction site were used in these assays. A fixed concentration of 40 nM FITC-labeled vIRF1 peptide was titrated with increasing concentrations of USP7-NTD (up to 500 μM) to detect the fluorescence polarization changes during formation of the protein-peptide complex. The dissociation constant of WT vIRF1 peptide was calculated to be 2.0 ± 0.1 μM, whereas the $K_d$ for the mutant peptide was calculated to be 46.1 ± 4.4 μM (Fig. 2, A and B). Interaction was not observed between USP7-NTD-DW and either WT or mutant vIRF1 peptides (Fig. 2, A and B). The $K_d$ value of 2 μM for the interaction between vIRF1 and USP7 compares well with that of EBNA1 and corroborates that KSHV vIRF1 interacts with USP7-NTD.

**NMR Analysis of the USP7-NTD and vIRF1 Peptide Interaction**—To further investigate the interaction between USP7-NTD and the vIRF1 peptide, two-dimensional NMR HSQC spectra of 15N-labeled USP7-NTD were analyzed in the presence of unlabeled WT (44SPGEPSPGTG53) or mutant (44SPGEPAGTG53) vIRF1 peptides. Fig. 2C displays the overlay of USP7-NTD 1H-15N HSQC spectra for USP7-NTD alone (in black) and USP7-NTD titrated with 0.4 mM of wild-type (in red) or mutant (in cyan) vIRF1 peptides (2:1 peptide:USP7-NTD ratio). Strong perturbations were observed in the USP7-NTD resonances in the presence of WT vIRF1 peptide, whereas these disturbances were notably decreased in the presence of mutant vIRF1 peptide. Using previously assigned USP7-NTD spectra (15) (Biological Magnetic Resonance Data Bank (BMRB) Entry 6939), we were able to observe that upon vIRF1 peptide binding, disturbances in USP7-NTD spectra were mostly in β-strand 7 residues such as Asp164, Trp165 (shift was observed in indole side chain), Gly166, Phe167, Ser168, and Met171 (Fig. 2D). Resonances of a few residues from β3, β4, and β6 were also affected including Met100, Phe118, and Ser155. These observations are very similar to those of USP7-NTD-EBNA1 interaction. However, in contrast to EBNA1, spectra of residues such as Asn162 and Phe170 from β7 and residues Met102 and Phe117 did not show any change upon the addition of vIRF1 peptide, suggesting a slightly different mode of interaction with USP7-NTD.

**Crystal Structure of the USP7-NTD-vIRF1 Peptide Complex**—To elucidate the molecular basis of this interaction in vitro, we co-crystallized USP7-NTD with the vIRF1 peptide, 44SPGEPSPGTG53. The structure of the USP7-NTD-vIRF1 peptide complex was determined using molecular replacement and refined to 1.5 Å resolution. USP7-NTD residues 54–62 and 106–111 are disordered and were not built in the final model. As shown previously, USP7-NTD forms a TRAF domain similar to that of tumor necrosis factor receptor-associated factor (TRAF) 2, which consists of an eight-stranded antiparallel β-sandwich fold with a shallow groove on the surface (Fig. 3, A and B) (38). The conserved 164DWGF167 motif is found in β-strand 7, which also contains a β-bulge essential for its interaction with binding partners. The electron density allowed building of vIRF1 residues (44SPGEPSPG51) in the binding site of USP7-NTD (Fig. 3, A and B). Interaction between USP7-NTD and vIRF1—Fluorescence polarization was used to determine the dissociation constant between USP7-NTD and vIRF1 peptides. FITC-labeled WT (44SPGEPSPGTG53) and mutant (44SPGEPAGTG53) vIRF1 peptides containing the potential USP7 interaction site were used in these assays. A fixed concentration of 40 nM FITC-labeled vIRF1 peptide was titrated with increasing concentrations of USP7-NTD (up to 500 μM) to detect the fluorescence polarization changes during formation of the protein-peptide complex. The dissociation constant of WT vIRF1 peptide was calculated to be 2.0 ± 0.1 μM, whereas the $K_d$ for the mutant peptide was calculated to be 46.1 ± 4.4 μM (Fig. 2, A and B). Interaction was not observed between USP7-NTD-DW and either WT or mutant vIRF1 peptides (Fig. 2, A and B). The $K_d$ value of 2 μM for the interaction between vIRF1 and USP7 compares well with that of EBNA1 and corroborates that KSHV vIRF1 interacts with USP7-NTD.
Residues 52 and 53 of the vIRF1 peptide did not have interpretable electron density, suggesting that they do not make contact with USP7-NTD. There are several polar and non-polar interactions that occur between USP7-NTD and the vIRF1 peptide (Fig. 3D). Ser50 of vIRF1 forms H-bonds through its side chain hydroxyl and backbone amide group with Asp164 of USP7-NTD. It also forms a H-bond with Arg104 of USP7-NTD. The Glu47 side chain of vIRF1 forms a water-mediated H-bond with Trp165 of USP7. Comparison between the EBNA1 and the vIRF1 peptides revealed that they are superimposable and make very similar contacts with USP7-NTD (Fig. 3E).

**USP7 and vIRF1 Interact in Vivo**—We investigated the vIRF1 interaction with USP7 in vivo. The ability of vIRF1 to interact with USP7 was examined by transfecting U2OS cells with Myc-tagged USP7 and FLAG-tagged WT vIRF1. After immunoprecipitation of the lysate with a USP7 antibody, immunoblotting with anti-FLAG led to identification of FLAG-tagged vIRF1 (Fig. 4A). Lysate incubated with rabbit IgG served as a negative control and did not show any interaction. We also examined whether the PEGPS vIRF1 deletion mutant (ΔvIRF1) could be co-immunoprecipitated with USP7. U2OS cells were transfected with Myc-tagged USP7 and FLAG-tagged ΔvIRF1. The lysate was immunoprecipitated with a USP7 antibody and immunobotted with anti-FLAG; however, ΔvIRF1 could not be detected (Fig. 4A). These results indicated that USP7 was interacting with vIRF1 but not ΔvIRF1 in U2OS cells.

We also performed the reciprocal experiments in which immunoprecipitation of the lysate with anti-FLAG rather than anti-USP7 readily identified USP7 in complex with vIRF1, whereas ΔvIRF1 was not able to pull down USP7 (Fig. 4B). Combined, these results confirmed that the EGPS residues in vIRF1 are essential for interaction with USP7 in vivo.
Blotting for anti-FLAG readily identified vIRF1 (Fig. 4C). In a reciprocal experiment, immunoprecipitation of FLAG-tagged vIRF1 from transfected U2OS lysate also successfully led to detection of endogenous USP7. In each case, lysate incubated with rabbit IgG served as negative control that did not show any interaction.

**Effect of vIRF1 on p53 Stability—**USP7 stabilizes cellular levels of the tumor suppressor, p53, and its negative regulator, Hdm2, through deubiquitination. Reduction in cellular levels of USP7 leads to instability of p53 (21), whereas deletion of USP7 leads to degradation of Hdm2, resulting in stabilization of p53 (23). We hypothesized that interaction between vIRF1 and USP7-NTD at the same binding site that is known to interact with p53 and Hdm2 should decrease the availability of USP7 and therefore lead to instability of p53. To test the effect of vIRF1 on p53, U2OS cells were transfected with expression vectors for FLAG-tagged vIRF1 or vIRF1. Endogenous levels of p53, Ser15-phosphorylated p53, and USP7 were detected by immunoblotting after transfection. As shown in Fig. 4, D and F, transfection of cells with vIRF1 resulted in a significant decrease in the level of p53 (about 50%) when compared with levels in cells transfected with empty vector (p < 0.01) or ΔvIRF1.

**Effect of vIRF1 on Hdm2 Stability—**Considering that USP7 deubiquitinates and stabilizes Hdm2, we monitored Hdm2 as well as phospho-Hdm2 (Ser166) in cells transfected with vIRF1 or ΔvIRF1 to assure that changes in the levels of p53 were not a result of change in the stability of Hdm2. Phosphorylation at Ser166 stabilizes Hdm2 and stimulates p53 ubiquitination (39). To test our hypothesis, U2OS cells were transfected with expression vectors for FLAG-tagged vIRF1 or ΔvIRF1. As shown in Fig. 4E, expression of vIRF1 or ΔvIRF1 had no effect on the endogenous levels of USP7, Hdm2, or Ser166-phosphorylated Hdm2 when compared with control. This observation suggests that change in the stability of p53 is not caused by changes in the stability of Hdm2 or USP7.

**Effect of vIRF1 on ATM Activity—**It was previously reported that vIRF1 expression inhibits auto-phosphorylation of ATM on Ser1981, which is important for ATM activation. Decreased ATM activity resulted in reduced Ser15 phosphorylation of p53 (11). p53 phosphorylation decreases the Hdm2 affinity for p53 and leads to the stability of p53 (40). As shown in Fig. 4D, we observed decreased levels of Ser1981-phosphorylated ATM as well as Ser15-phosphorylated p53 in cells transfected with vIRF1 or ΔvIRF1, suggesting that under our experimental conditions, ΔvIRF1, which is incapable of binding to USP7, is still able to exert an inhibitory effect on ATM activation and Ser15 phosphorylation of p53. This indicates that the reduced p53 levels observed with ΔvIRF1 when compared with vIRF1 are mainly due to the interaction between vIRF1 and USP7. Therefore, we show that interaction of vIRF1 with USP7 leads to destabilization of p53.

**Discussion**

Members of the herpesvirus family such as herpes simplex virus type 1, Epstein-Barr virus, Kaposi sarcoma herpesvirus, and cytomegalovirus have evolved to interfere with the USP7-p53-Hdm2 pathway by competitively binding to USP7 and hindering its interaction with cellular substrates or binding proteins (14, 15, 25, 34, 35, 41). HSV-1 ICP0 is an E3 ubiquitin ligase that ubiquitinates and causes degradation of host proteins (42). ICP0 interaction with USP7 leads to its rescue from self-ubiquitination and proteasomal degradation (43). UL35, a human CMV latent protein, interacts with USP7 and alters its subcellular localization (41). UL35 also co-localizes with and disrupts promyelocytic leukemia (PML) nuclear bodies. Other USP7-interacting viral proteins such as ICP0 and EBNA1 also disrupt PML nuclear bodies and induce degradation of PML.
proteins (44, 45). PML nuclear bodies are primarily composed of PML protein and are important mediators of critical cellular processes including apoptosis, DNA repair, and intrinsic response to viral infection (41, 46, 47). PML nuclear bodies also recruit proteins such as p53 and p300, which interact with vIRF1 (9, 48). In KSHV-infected BCBL-1 cells, vIRF1 was shown to localize with PMLs during both lytic and latent cycles (49). USP7 also associates with PML nuclear bodies (44, 50).

Through analysis of viral protein sequences followed by biochemical, structural, and in vivo studies, we identified KSHV vIRF1 as a novel USP7-binding protein. Fluorescence polarization indicated that the vIRF1 peptide, 44SPGEGPSGTG53, interacts with USP7-NTD with a $K_d$ value of 2.0 $\mu M$ when compared with substrates such as Hdm2 and p53 that bind with $K_d$ values varying between 10 and 45 $\mu M$, respectively. A single point mutation of Ser to Ala in the vIRF1 peptide decreased the
FIGURE 5. A new role for vIRF1 in hijacking USP7 and degrading p53. vIRF1 disrupts the p53 signaling pathway during viral infection by 1) inhibiting ATM phosphorylation, 2) inhibiting p53 transcription activation, and 3) inhibiting p300 acetylation of p53. Our results suggest that vIRF1 also binds USP7 and decreases the availability of USP7 for deubiquitinating and stabilizing p53.
dissociation constant ~25-fold, indicating the importance of Ser\(^{50}\) in mediating interaction with the USP7-NTD binding site. The interaction between USP7-NTD\(^{138}\) and the vIRF1 peptide was completely abolished, confirming that the vIRF1 \(^{47} EGPS \) sequence interacts with the USP7-NTD\(^{164}\)DWGF\(^{167}\) motif.

The vIRF1 peptide binds to a groove on the surface of USP7-NTD, forming identical interactions with USP7-NTD as those seen with the USP7-NTD-EBNA1 peptide complex structure. The HSQC NMR analysis revealed perturbations in USP7-NTD residues from \(\beta\)-strands 3, 4, 6, and 7 upon the addition of the vIRF1 peptide. These residues correlate well with the residues seen in the crystal structure analysis, especially those found in \(\beta\)-strand 7. The side chains of residues from \(\beta\)-strands 3 and 7 form polar contacts with the vIRF1 peptide, whereas the remaining residue side chains, from \(\beta\)-strands 4 and 6, are in close proximity to the vIRF1 peptide but do not make any polar contacts. Along with EBNA1 and ORF45, vIRF1 contains a negatively charged glutamic acid residue at the position of P/A in the (P/A)XXS USP7 binding motif. Glu rather than Pro or Ala in that position is thought to increase the affinity of EBNA1 for USP7-NTD, therefore allowing it to effectively compete with USP7 cellular substrates \(^{18}\). Comparing the interaction with USP7-NTD made by Glu rather than Pro or Ala shows an increased number of H-bonds formed with Glu to USP7-NTD. This unique mode of interaction achieved through the substitution of a Glu residue for Ala or Pro is advantageous for vIRF1, ORF45, and EBNA1 in that it provides them with a higher binding affinity. This empowers the virus to sufficiently disrupt USP7 deubiquitination of cellular substrates such as p53. Identification of yet another USP7-NTD-interacting herpesvirus protein suggests that these viruses target USP7 at least in part because it is a critical cellular regulator of p53.

Our \textit{in vivo} data in U2OS cells indicate that vIRF1, but not \(\Delta vIRF1\), leads to a decrease in the levels of cellular p53. Therefore, vIRF1 binds USP7-NTD through its EGPS sequence and decreases the availability of USP7 for binding and deubiquitinating p53. vIRF1 was previously reported to directly interact with p53 through its central region (residues 152–360), prevent p53 acetylation by p300, and thus inhibit p53 transcriptional activation \(^{11, 48}\). It was also reported that vIRF1 led to increased ubiquitination and degradation of p53 \(^{11}\). Our data suggest that increased ubiquitination and degradation of p53 can in part be attributed to vIRF1 hijacking USP7, thus decreasing the ability of USP7 to deubiquitinate and stabilize p53.

Because Hdm2, a p53 negative regulator, is also a substrate of USP7, we monitored cellular levels of Hdm2 to investigate whether the vIRF1-mediated decrease in the availability of USP7 also destabilized Hdm2. We were never able to detect any significant changes in the level of Hdm2, suggesting that the USP7-vIRF1 interaction preferably disrupted USP7 deubiquitination and stabilization of p53.

It was previously reported that vIRF1 inhibits Ser\(^{1981}\) autophosphorylation (activation) of ATM, which prevents ATM-mediated Ser\(^{15}\) phosphorylation of p53 and leads to p53 degradation \(^{11}\). We monitored ATM and Ser\(^{1981}\)-phosphorylated ATM but did not observe any changes in their levels between vIRF1- and \(\Delta vIRF1\)-transfected cells; however, both showed lower levels when compared with mock-transfected cells, indicating that vIRF1 and \(\Delta vIRF1\) are still able to interact with ATM and inhibit its activity. As it was previously reported that vIRF1 interacts with ATM through its CTD, whereas the USP7 binding motif that we identified is located N-terminal to its DBD, we were not expecting changes in ATM levels \(^{11}\).

High levels of vIRF1 expression in KSHV-infected BCBL-1 cells were only observed transiently during lytic infection \(^{49}\). However, low levels of vIRF1, localized with PML nuclear bodies, were reported during latency \(^{49}\). In KSHV-infected cells, p53 was only detectable in PML nuclear bodies during the lytic cycle, whereas latently infected cells did not show detectable levels of p53 \(^{49}\). These observations suggest that vIRF1 may prevent USP7 deubiquitination of p53 during latency as all three proteins are localized to PML nuclear bodies. However, it is also important that vIRF1-mediated disruption of USP7 regulation of p53 is only one of the many mechanisms used by vIRF1 and other KSHV-expressed proteins to weaken the cellular antiviral response \(^{4}\).

KSHV vIRF4, ORF45, and LANA interact with and inhibit multiple members of the USP7-p53-Hdm2 pathway, indicating the importance of p53 degradation for KSHV to establish and maintain life-long latency in host cells \(^{11, 34, 35, 51}\). vIRF1 also uses multiple redundant mechanisms to combat the cell’s antiviral response by inhibiting multiple targets of the USP7-p53-Hdm2 pathway. Under normal (unstressed) cellular conditions, USP7 predominantly stabilizes Hdm2, which permits p53 ubiquitination and degradation as low levels of p53 are required for normal cellular homeostasis (Fig. 5). Upon cellular stress (DNA damage or viral entry into the nucleus), ATM kinase is activated, which leads to phosphorylation of p53, Hdm2, and HdmX. ATM-mediated Hdm2 phosphorylation leads to p53 stabilization. ATM-mediated phosphorylation of p53 decreases its affinity for Hdm2 and signals its acetylation and transactivation by p300, as well as its stabilization by USP7. vIRF1 is a potent inhibitor of ATM kinase activity, p300 acetyltransferase activity, and p53 transcriptional activity (Fig. 5). We have shown that vIRF1 interaction with USP7 decreases p53 levels, suggesting that it also blocks USP7 deubiquitination and stabilization of p53. Our novel finding that vIRF1 also targets USP7 fits well with the mechanism that KSHV must destroy p53 for a successful lifelong infection.

\textbf{Author Contributions—}S. C. designed and performed biochemical and cell biological experiments. I. K. L. analyzed data and prepared the mechanism. O. E. performed initial cell biology experiments. S. F. and Y. S. performed and analyzed NMR experiments. V. S. conceived, designed, supervised, the study, determined the crystal structure, and wrote the manuscript with input from other authors. All authors analyzed the results and approved the final version of the manuscript.

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Crystal Structure of the USP7-ntvIRF1 Peptide Complex

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