Title: Inhibition of RIG-I mediated signaling by Kaposi’s sarcoma-associated herpesvirus-encoded deubiquitinase ORF64

Running title: Inhibition of RIG-I by KSHV ORF64

Kyung-Soo Inn, Sun-Hwa Lee, Jessica Y. Rathbun, Lai-Yee Wong, Zsolt Toth,
Keigo Machida, Jing-Hsiung James Ou, Jae U. Jung*

Department of Molecular Microbiology and Immunology, University of Southern California,
Keck School of Medicine, Los Angeles, CA 90033, USA

*: Correspondence:
Jae U Jung <jaeujung@usc.edu>
Department of Molecular Microbiology and Immunology
University of Southern California
Keck School of Medicine
Los Angeles, CA 90033, USA
Phone: 323-442-1713
Fax: 323-442-1721

Word Count
Abstract: 98
Main Text (excluding References and Figure Legends): 1683

Copyright © 2011, American Society for Microbiology and/or the Listed Authors/Institutions. All Rights Reserved.
Abstract

Virus infection triggers interferon (IFN)-mediated innate immune defenses in part through viral nucleic acid interactions. However, the immune recognition mechanisms by which the host identifies incoming DNA viruses are still elusive. Here, we show that increased levels of Kaposi's sarcoma-associated herpesvirus (KSHV) persistency are observed in Retinoic acid-inducible gene I (RIG-I) deficient cells and that KSHV ORF64, a tegument protein with deubiquitinase (DUB) activity, suppresses RIG-I-mediated IFN signaling by reducing the ubiquitination of RIG-I, crucial for its activation. This study suggests a potential role of RIG-I in sensing KSHV infection and that KSHV ORF64 DUB counteracts RIG-I signaling.
Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8) belongs to the gammaherpesvirus family, which includes Epstein-Barr virus (EBV), murine gammaherpesvirus-68 (MHV68), and Herpesvirus Saimiri (HSV). KSHV is etiologically linked to Kaposi’s sarcoma (KS) as well as two rare B-cell proliferative diseases, Primary Effusion Lymphoma (PEL) and Multicentric Castlemen’s Disease (MCD) (3, 4). Recent studies have broadened our understanding of the mechanisms by which a number of innate immune sensors recognize herpesviruses and thereby induce innate immune responses (reviewed in 7, 21). Specifically, Toll-like receptor (TLR) 3 and 4 are involved in antiviral responses against KSHV (17, 32). Moreover, IFN-α treatment has been shown to suppress KSHV lytic replication in PEL cells (20), and higher MHV68 reactivation in IFN-α/β receptor deficient cells compared to wild-type cells has been reported (2). These studies suggest that IFN plays an important role in antiviral responses against gammaherpesviruses. To maintain persistent infection in hosts, KSHV employs a battery of genes (e.g. vIRFs, RTA, ORF45, and K-bZIP) to antagonize type-I IFN signaling (reviewed in 7, 18). However, the precise details of how host intracellular pattern recognition receptors such as RIG-I-like receptors (RLRs) function in the recognition of incoming DNA viruses and how herpesviruses evade host RLR-mediated detection remain elusive.

RIG-I is a cytosolic RNA sensor that recognizes viral RNA and is subsequently ubiquitinated by Tripartite motif-protein 25 (TRIM25) (10) to initiate the IFN signaling cascade. Recently, a growing body of evidence suggests that RIG-I is not only involved in the recognition of RNA viruses, but also DNA viruses including herpesviruses, further highlighting the importance of RIG-I as a viral sensor (1, 5, 6, 22-24). In order to investigate the role of RIG-I-mediated IFN signaling upon KSHV infection, we used a recombinant virus, rKSHV.219, that
expresses the red fluorescent protein (RFP) through the KSHV lytic PAN promoter and the green fluorescent protein (GFP) through the EF-1α promoter (29). 293A cells carrying rKSHV.219 were mock treated or infected with Sendai virus (SeV) (100 HAU/ml), and then used for quantitative RT-PCR (qRT-PCR) to measure IFN-β mRNA. This showed that IFN-β mRNA levels were much lower in 293A.rKSHV.219 cells infected with SeV than in naïve 293A cells (Fig. 1A). The induction of KSHV lytic replication with sodium butyrate (NaB) treatment resulted in a minimal induction of IFN-β expression in 293A.rKSHV.219 cells compared to naïve 293A cells (Fig. 1A). By contrast, transfection of total RNAs purified from Vero cells carrying lytically replicating KSHV, but not from Vero cells carrying latent KSHV, readily induced IFN-β promoter activation upon RIG-I over-expression (Fig. 1B), suggesting that RIG-I is capable of recognizing RNAs derived from KSHV replication to induce IFN signaling.

A number of viruses encode deubiquitination enzymes (DUBs) to manipulate cellular processes and several virally encoded DUBs have been shown to antagonize IFN signaling (13, 28, 30, 34). Recently, a number of studies have shown herpesviridae have large tegument proteins with a DUB domain embedded at their N-terminus, such as herpes simplex virus-1 (HSV-1) UL36USP, mouse cytomegalovirus (MCMV) M48, human cytomegalovirus (HCMV) UL48, EBV BPLF1, and KSHV and MHV68 ORF64 (11, 14, 25, 26, 31). These viral tegument DUBs can cleave either Lys48- or Lys63-linked polyubiquitin chains in in vitro deubiquitination assays (11, 16, 26, 33). Since ubiquitination plays a critical role in RIG-I signaling (10), we tested whether KSHV ORF64 inhibits RIG-I function. Ectopic expression of KSHV ORF64 (kindly gifted from Dr. Damania) readily suppressed RIG-I-2CARD-induced IFN-β promoter activation (Fig. 1C), as well as SeV infection-induced IFN-β promoter activation (Fig. 1D). However, the KSHV ORF64-C29G mutant, which is enzymatically defective due to the mutation
of its conserved cysteine (11), showed much weaker or no inhibition, suggesting that DUB activity is required for ORF64 to inhibit RIG-I-2CARD- or SeV infection-induced IFN-β promoter activation (Fig. 1C and D). Moreover, KSHV ORF64 blocked IFN-β and IFI56 mRNA production induced by SeV infection, whereas the ORF64-C29G mutant did not (Fig 1E and F).

As seen with KSHV ORF64, MHV68 ORF64 was also able to inhibit RIG-I-2CARD- and SeV-induced IFN-β promoter activation, indicating that the ability of this tegument DUB to inhibit RIG-I function is conserved between KSHV and MHV68 (Fig. 1G and H).

Upon viral infection, the N-terminal CARDs of RIG-I undergo K$_{63}$-linked ubiquitination induced by E3 ligase TRIM25 that is critical for its interaction with its downstream signaling partner MAVS/VISA/IPS-1/Cardif, which ultimately leads to RIG-I mediated IFN responses (15, 19). Interestingly, despite its robust ability to block RIG-I function, ORF64 was unable to suppress MAVS-mediated activation of IFN-β, NF-κB, and ISRE promoter activities (Fig. 2A).

In contrast, the KSHV RTA and vIRF2 proteins were able to suppress both RIG-I-2CARD and MAVS induction of IFN-β promoter activation (Fig. 2B), indicating that as previously shown (8, 35), RTA and vIRF2 target distinct steps of the IFN pathway downstream of RIG-I and MAVS.

To test whether KSHV utilizes ORF64 DUB activity to specifically target RIG-I ubiquitination and thereby block IFN signaling, we examined the levels of RIG-I-2CARD ubiquitination upon co-expression with ORF64 WT or its C29G mutant in 293T cells. vIRF2 was included as a negative control. Ectopic expression of ORF64 WT severely reduced the ubiquitination of RIG-I-2CARD, whereas the ORF64-C29G mutant did not do so under the same conditions (Fig. 2C and D). Furthermore, treatment of MG132 proteosomal inhibitor (20 μM for 4h) showed little or no effect on GST-RIG-I-2Card levels, indicating that ORF64 markedly downregulates RIG-I ubiquitination without affecting its expression. By contrast, vIRF2 showed any effect on RIG-I-
2-CARD ubiquitination (Fig. 2C). Furthermore, due to its efficient down-regulation of RIG-I ubiquitination that is essential for its interaction with the downstream MAVS signaling molecule, KSHV ORF64 effectively blocked the interaction between RIG-I-2CARD and MAVS-CARD (Fig. 2E). Finally, the inhibitory effects of ORF64 on RIG-I-mediated induction of IFN signaling were partially reversed by the overexpression of TRIM25; ectopic expression of WT TRIM25, but not E3 ligase mutant TRIM25 RING\textsuperscript{CS} (10), restored not only RIG-I ubiquitination but also RIG-I-mediated induction of IFN-β promoter activity (Fig. 2F and 2G). These results demonstrate that KSHV ORF64 targets TRIM25-mediated RIG-I ubiquitination, contributing to the inhibition of IFN signaling.

Since KSHV utilizes ORF64 DUB activity to specifically counteract RIG-I-mediated IFN signaling, we then examined the exact role of RIG-I in KSHV infection and replication. To this end, we utilized Huh7 human hepatocellular carcinoma cells and its derivative, Huh7.5 cells carrying RIG-I with a T\textsubscript{55}I mutation at its 1\textsuperscript{st} CARD that abolishes TRIM25 interaction and thereby disrupts the signaling function of RIG-I to induce anti-viral IFN production, leading to a high permissiveness to HCV replication (9, 27). At 24h post-infection with rKSHV.219, significantly higher GFP signal, a marker of rKSHV.219 infection, was observed in Huh7.5 cells compared to Huh7 cells (Fig. 3A). While the loss of intracellular KSHV DNA content was observed in both infected Huh7 and Huh7.5 cells over a 24h incubation period, it was markedly faster in Huh7 cells compared to Huh7.5 cells (Fig. 3B). qRT-PCR revealed that the levels of viral transcripts including LANA (latent gene), RTA (immediate-early gene) and ORF25 (late gene) were considerably higher in Huh7.5 cells than in Huh7 cells (Fig. 3C). Similarly, recombinant MHV68-GFP replicated in \textit{RIG-I}\textsuperscript{-/-} mouse embryonic fibroblasts (MEFs) at markedly higher levels than in \textit{RIG-I}\textsuperscript{+/+} MEFs: \textit{RIG-I}\textsuperscript{-/-} MEFs showed ~230-fold higher virus
loads at M.O.I of 2 compared to RIG-I+/- MEFs (Fig. 3D and E). In contrast, the supplementation of RIG-I+/- MEFs with wild-type RIG-I led to the significant reduction of MHV68-GFP replication (Fig. 3D and E). These results indicate a potential role of RIG-I in recognizing incoming gammaherpesviral DNAs.

Next, the effect of RIG-I activity on KSHV lytic reactivation was examined by the shRNA-mediated depletion of RIG-I expression (Open biosystems; RHS3979-99220020) in 293A.rKSHV.219 cells. RIG-I depletion resulted in the increased RTA, and ORF25 mRNA levels with or without NaB treatment (Fig. 4A). The RIG-I splicing variant (RIG-I SV), that has a short deletion (amino acids 36-80) within its 1st CARD, not only loses TRIM25-mediated ubiquitination, but also effectively suppresses RIG-I-mediated IFN-β production (9). RIG-I SV was transfected into 293A.rKSHV.219 cells, followed by NaB (3mM) treatment to induce the lytic reactivation of rKSHV.219. This showed that ectopic expression of RIG-I SV pronouncedly increased the numbers of RFP-positive cells, a marker for rKSHV.219 reactivation (Fig. 4B). Correlated with this, the levels of RTA and ORF25 transcripts were detectably higher in cells transfected with RIG-I SV compared to cells transfected with vector alone (Fig. 4C). Collectively, these results suggest that RIG-I-mediated antiviral signaling is involved in the suppression of KSHV lytic reactivation.

The discovery of an Ub-specific cysteine protease encoded within the HSV-1 UL36 tegument protein was a seminal finding (14). Since then, a number of herpesvirus ubiquitin-specific proteases have been studied for their functions in the viral lifecycle (11, 25, 26, 31). Specifically, recombinant MHV68 carrying an enzymatically inactive ORF64 was cleared faster than revertant viruses in an in vivo mouse infection model (12), a HCMV UL48 mutant virus produced 10-fold lower progeny virus (16), and KSHV ORF64 depletion resulted in decreased
viral lytic transcription and lytic protein expression (11). Here, we demonstrate that increased levels of KSHV persistency are observed in RIG-I deficient or depleted cells, and that the KSHV ORF64 DUB enzyme specifically targets and suppresses RIG-I mediated signaling, suggesting a potential role of RIG-I in detecting KSHV infection and that KSHV ORF64 counteracts RIG-I signaling. However, it remains elusive what component(s) of KSHV is sensed by RIG-I. It has been reported that EBER1 and EBER2, the most abundant non-coding RNAs produced during EBV infection, activate the RIG-I pathway (23). However, we found that unlike the EBV EBER1/2, the KSHV PAN RNA (T1.1/nut-1), the most abundant non-coding RNA produced during KSHV infection (36, 37), did not induce RIG-I activation (data not shown). Further study is thus necessary to elucidate how RIG-I senses KSHV infection. Finally, since another tegument protein, ORF45 encoded by KSHV has been shown to block host IFN signaling by inhibiting IRF7 activity (38, 39), KSHV utilizes two virion-associated ORF45 and ORF64 tegument proteins to mitigate intracellular RIG-I-mediated antiviral responses to enhance KSHV infectivity and persistency.

This work was partly supported by CA82057, CA91819, CA31363, CA115284, CA147868, CA148616, DE019085, AI073099, AI083025, Hastings Foundation, Fletcher Jones Foundation National Agenda Project grant from Korea Research Council of Fundamental Science & Technology. We thank Dr. Blossom Damania for reagents and Stacy Lee for manuscript preparation.
References


Figure Legends

Figure 1. Inhibition of RIG-I-mediated signaling by KSHV ORF64. (A) 293A and 293A-rKSHV.219 cells were mock-treated or treated with sodium butyrate (NaB; 3mM) to induce lytic replication. After Sendai virus (SeV) infection, IFN-β mRNA levels were analyzed by qPCR. Values were normalized to U6 RNA transcript. ND: Non-detectable. (B) Vero or Vero-rKSHV.219 (219) cells were mock-treated or treated with sodium butyrate (NaB) to induce lytic reactivation. Total RNAs were prepared from cells using TRIzol, followed by treatment of DNase I. Purified total RNAs were transfected with reporter plasmids into HEK293T cells and subjected to IFN-β promoter luciferase assay. (C-D) Effects of KSHV ORF64 and ORF64-C29G mutant on IFN-β promoter activity induced by RIG-I-2CARD (C) or SeV infection (D) were analyzed by dual-luciferase assay. Values were normalized by TK-Renilla luciferase values. P-value was calculated by two-tailed t-test. (E-F) Effects of KSHV ORF64 and ORF64-C29G (C29G) mutant on IFN-β (E) and IFI56 (F) transcription upon SeV infection were analyzed by qRT-PCR. (G-H) Effect of MHV68 ORF64 on IFN-β promoter activity induced by RIG-I-2CARD (G) or SeV infection (H) was analyzed by dual-luciferase assay. P-values were calculated by two-tailed t-test. Expression of ORF64 and ORF64-C29G mutant was confirmed by immunoblot assays (C-H).

Figure 2. Reduction of RIG-I ubiquitination induced by KSHV ORF64. (A) HEK293T cells were transfected with vector, RIG-I-2CARD or MAVS together with vector or KSHV ORF64. IFN-β, NF-κB, and ISRE promoter activities were assessed by dual-luciferase assays. (B) Vector, ORF64, RTA or vIRF2 was transfected together with RIG-I-2CARD and reporter
plasmids. IFN-β promoter activity was assessed by dual-luciferase assay. (C) GST-RIG-I-2CARD was transfected with vector, ORF64 (WT), or vIRF2 into HEK293T cells. RIG-I-2CARD ubiquitination was analyzed by GST-pulldown (GST-PD), followed by immunoblotting (IB) with antibodies as indicated. Whole cell lysates (WCL) were analyzed by IB to show expression levels. (D) GST-RIG-I-2CARD was transfected with vector, ORF64 (WT), or ORF64-C29G mutant (C29G) into HEK293T cells, followed by treatment with DMSO or MG132 (20 µM) for 4 hours before harvest. RIG-I-2CARD ubiquitination was analyzed as described in (C). (E) GST-RIG-I-2CARD and Flag-MAVS-CARD were transfected with vector, ORF64 (WT), or ORF64-C29G mutant (C29G) into HEK293T cells as indicated. Cell lysates were subjected to GST-PD, followed by IB with antibodies as indicated. (F) GST-RIG-I-2CARD was transfected with vector or ORF64. TRIM25 (WT) or TRIM25-RINGCS mutant (RINGCS) were transfected together as indicated. RIG-I-2CARD ubiquitination was analyzed as described in (C). (G) Effect of TRIM25 on ORF64 was analyzed by luciferase assay. Plasmids were transfected as indicated. Experimental procedures were as described in (A).

Figure 3. Increased susceptibility of RIG-I deficient cells to KSHV and MHV68 infection.

(A) Huh7 and Huh7.5 cells were infected with rKSHV.219 virus at multiplicity of infection (M.O.I) of 2. Pictures were taken at 24h after infection. (B) Intracellular KSHV DNA amounts. At indicated time points after rKSHV.219 infection, total DNAs were purified using a genomic DNA purification kit and KSHV DNA amounts were measured by qPCR using a RTA primer set. Values were normalized to actin promoter DNA value. DNA amount at 1 hour after infection is set to 100%. (C) The mRNA levels of LANA, RTA and ORF25 were measured from mock-infected or KSHV infected Huh7 and Huh7.5 cells by qPCR. Values were normalized to U6.
housekeeping gene transcript. ND: Non-detectable. (D) RIG-I+/+, RIG-I−/− and RIG-I reconstituted RIG-I−/− (RIG-I−/−-WT-RIG-I) MEFs were infected with MHV68-GFP (m.o.i = 0.2).

(E) Viral titers of MHV68-GFP from infected RIG-I+/+, RIG-I−/− and RIG-I reconstituted RIG-I−/− (RIG-I−/−-WT-RIG-I) MEFs were determined by plaque assay.

Figure 4. Increased lytic replication of KSHV by the suppression of RIG-I function or expression. (A) 293A.rKSHV.219 cells were transfected with control or RIG-I silencing shRNAs. Cells treated with NaB for 48h were subjected to qRT-PCR analysis to measure the mRNA levels of viral and RIG-I genes. Values were normalized to actin mRNA. (B) 293A.rKSHV.219 cells were transfected with vector or RIG-I splicing variant (RIG-I SV) and treated with sodium butyrate (NaB; 3mM) to induce lytic replication. Numbers of RFP-positive cells were counted and presented as a bar graph (right panel). (C) At 48 h after NaB treatment, the levels of LANA, RTA and ORF25 mRNA were measured from cells transfected with either vector or RIG-I SV by qRT-PCR. Values were normalized to actin mRNA. P-values were calculated by two-tailed t-test.
Figure 3.
Figure 4.