# A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation

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Edited by George Klein, Karolinska Institute, Stockholm, Sweden, and approved February 14, 2002 (received for review August 9, 2001)

**Interferons constitute the earliest immune response against viral infection. They elicit antiviral effects as well as multiple biological responses involved in cell growth regulation and immune activation. Because the interferon-induced cellular antiviral response is the primary defense mechanism against viral infection, many viruses have evolved strategies to antagonize the inhibitory effects of interferon. Here, we demonstrate a strategy that Kaposi's sarcoma-associated herpesvirus uses to block virus-mediated induction of type I interferon. We found that a viral immediate-early protein, namely ORF45, interacts with cellular interferon-regulatory factor 7 (IRF-7). In consequence, IRF-7 phosphorylation is inhibited and the accumulation of IRF-7 in the nucleus in response to viral infection is blocked. IRF-7 is a transcription regulator that is responsible for virus-mediated activation of type I interferon genes. By blocking the phosphorylation and nuclear translocation of IRF-7, ORF45 efficiently inhibits the activation of interferon $\alpha$ and $\beta$ genes during viral infection. Inhibition of interferon gene expression through a viral protein blocking the activation and nuclear translocation of a crucial transcription factor is a novel mechanism for viral immune evasion.**

Interferons (IFNs) are a family of multifunctional cytokines that constitute the earliest immune response against viral infection. They elicit antiviral effects and multiple biological responses such as activation of natural killer (NK) cells and macrophages, up-regulation of expression of MHC class I molecules, and protection of $CD8^+$ cells from antigen-induced cell death. There are two types of IFN, type I (IFN-$\alpha/\beta$) and type II (IFN-$\gamma$). The type I IFNs are produced by virus-infected cells and constitute the primary response against viral infection, whereas type II IFN is a Th1 cytokine produced by activated T cells and natural killer cells and is involved in immune regulation (1, 2). The induction of IFN is highly regulated through the IFN-regulatory factors (IRFs), a growing family of transcription factors with a broad range of activities (3–5). Within the IRF family, IRF-3 and IRF-7 have been identified as key regulators for the induction of type I IFNs (4, 5). IRF-3 is expressed constitutively in the cytoplasm in a variety of tissues. Upon viral infection, IRF-3 is activated through phosphorylation of specific residues near its C terminus. The phosphorylation results in conformational changes, including the formation of homodimers, and consequently leads to translocation of IRF-3 from the cytoplasm to the nucleus. In the nucleus, IRF-3 assembles with other transcription factors and contributes to the induction of specific defense genes, including IFN-$\beta$ (6–8). IRF-7 was originally identified as a protein that binds and represses the Epstein–Barr virus Qp promoter for Epstein–Barr virus nuclear antigen 1 (EBNA-1) (9). Soon after, it was shown to be an important component in IFN-$\alpha$ induction (10–12). IRF-7 is primarily expressed in cells of lymphoid origin at a low level. Its expression is stimulated by IFN, lipopolysaccharide, and viral infection. Like IRF-3, IRF-7 also undergoes virus-induced phosphorylation and nuclear translocation (10, 11). Interestingly, ectopic expression of IRF-7 can reconstitute virus-mediated IFN-$\alpha$ production in fibroblast cells, suggesting that IRF-7 is a critical determinant for the induction of IFN-$\alpha$ genes in response to viral infection (13).

Because the IFN-induced cellular antiviral response is the primary defense mechanism against viral infection, many viruses have evolved mechanisms to counteract IFN effects (2, 14). Some viruses were found to have the ability to block IFN signaling pathways. The most common target of viral action is the IFN-induced, double-stranded RNA-activated protein kinase (PKR), whose activation results in inhibition of protein synthesis (2). Recently, strategies for blocking IFN-$\alpha/\beta$ production through viral products targeting IFN-regulatory factors began to be unveiled. These viral products include E6 protein of human papillomavirus (15), NS1 protein of influenza A virus (16), E3L of vaccinia virus (17), and vIRF-1 of Kaposi's sarcoma-associated herpesvirus (KSHV) (18, 19). However, the mechanisms underlying these viral proteins blocking the activities of these IRFs remain undefined.

KSHV, also referred to as human herpevirus-8 (HHV-8), is a newly identified herpesvirus (20). This virus is associated with Kaposi's sarcoma (21), primary effusion lymphoma (PEL) (22), and multicentric Castleman's disease (MCD) (23). As a gammaherpesvirus, KSHV characteristically establishes latent infection in lymphoid cells. When latency is disrupted, the virus can switch to a lytic life cycle and express its lytic genes in a temporal and sequential order (24, 25). A few viral genes are expressed independently of *de novo* protein synthesis, and are classified as immediate-early genes. In general, immediate-early genes encode regulatory proteins that are crucial for primary infection and viral reactivation. Recently, we identified four immediate-early genes in the KSHV genome in viral reactivation (26). The aim of this study was to explore the functional roles of KSHV immediate-early genes in viral infection and reactivation. We found that one of KSHV immediate-early proteins, namely ORF45, interacts with cellular IRF-7 and blocks virus-mediated phosphorylation and nuclear translocation of IRF-7. In consequence, ORF45 efficiently inhibits virus-induced production of type I IFN.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: KSHV, Kaposi's sarcoma-associated herpesvirus; IFN, interferon; IRF-7, IFN-regulatory factor 7; GST, glutathione *S*-transferase; GFP, green fluorescent protein; RT-PCR, reverse transcription-coupled–polymerase chain reaction.

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## Materials and Methods

**Yeast Two-Hybrid Screening.** A yeast two-hybrid screen was performed with the Matchmaker system (CLONTECH). The KSHV ORF45 bait plasmid was constructed by cloning the full-length coding sequence of ORF45 in pAS2–1 in frame with GAL4 DNA-binding domain (DBD). The yeast strain Y190 carrying the plasmid pAS2–1-ORF45 was used to screen a human lymphocyte Matchmaker cDNA library (CLONTECH). One million transformants were plated on $Leu^-$ $Trp^-$ $His^-$ plus 50 mM 3-amino-1,2,4-triazole (3-AT) plates. $His^+$ $LacZ^+$ colonies were picked up for sequencing and further analyses.

***In Vitro* Binding Assay.** IRF-7 cDNA sequence obtained from the yeast two-hybrid screening was cloned into pGEX-5X-2 vector and glutathione *S*-transferase (GST)-IRF-7 fusion protein was synthesized and purified by glutathione-agarose beads. The full length of ORF45 and a series of truncated ORF45 mutants were cloned into pTM, a vector optimized for *in vitro* translation. ORF45 protein and its variants were synthesized *in vitro* by using the TNT coupled T7 transcription-translation system (Promega) in the presence of [$^{35}$S]methionine, and mixed with GST or GST-IRF-7 immobilized on glutathione-agarose. Complexes were washed three times with PBS and resolved by SDS/PAGE.

**Antibodies and Immunofluorescence Assay.** Polyclonal anti-ORF45 antibody was generated with ORF45 protein prepared by using a recombinant baculovirus system. Anti-Flag monoclonal antibody was purchased from Sigma. The secondary fluorochrome-conjugated antibodies were purchased from Vector Laboratories.

293T cells grown on coverslips were cotransfected with pCR3.1-ORF45 and pCMV-Flag-IRF7A. Twenty-four hours later, the cells were fixed with cold ethanol/acetone (50:50 vol/vol) and stained with anti-ORF45 rabbit polyclonal antibody (1:100) and anti-Flag (1:100) mouse monoclonal antibody. FITC-conjugated anti-mouse secondary antibody and Texas-red-conjugated anti-rabbit secondary antibody were used at a dilution at 1:100. The coverslips were mounted on slides with Vectashield (Vector Laboratories) and examined under an Olympus confocal microscope.

***In Vivo* Interaction Assay.** 293T cells were cotransfected with pCR3.1-ORF45 and pCMV-Flag-IRF7A by using a Qiagen Effectene transfection kit. At 24 h after transfection, cell extract was prepared and subjected to immunoprecipitation using anti-Flag affinity agarose (Sigma). Precipitates were washed two times with lysis buffer and an additional two times with PBS. Samples were boiled in SDS gel loading buffer and resolved by SDS/PAGE, followed by Western blot analysis using anti-Flag (1:5000) and anti-ORF45 (1:2000) antibodies. The ECL chemiluminescence system (Amersham Pharmacia) was used for detection on Western blots.

**Subcellular Localization Analysis.** Green fluorescent protein (GFP)-IRF-7 and GFP-ORF45 were constructed by cloning full-length cDNAs of IRF-7A and ORF45 into pEGFPN1 (CLONTECH). 293T cells grown in 24-well plates were transfected with pEGFP-IRF7A and pEGFP-ORF45 constructs, respectively, by using a Qiagen Effectene transfection kit. For virus infection, the transfected cells were challenged with Sendai virus (80 hemagglutinin units/ml; Safas, Storrs, CT) at 16 h after transfection. GFP fluorescence was analyzed in living cells at 4 and 12 h after infection with an Olympus confocal microscope with a 60× objective.

**Luciferase Assay.** The promoter sequences of human *IFNA1* (−140 to +9) and *IFNB* (−280 to +20) were cloned into pGL3-basic vector (Promega). Subconfluent 293T cells grown in 24-well plates were transfected with 10 ng of pRL-TK reporter (*Renilla* luciferase as an internal control), 100 ng of pGL-3 reporter (firefly luciferase, experimental reporter), 20 ng of pCR3.1-IRF7A, and increasing amounts (20–300 ng) of pCR3.1-ORF45 by using a Qiagen Effectene transfection kit. Blank plasmid pCR3.1 was added to samples to equalize the amount of DNA in each transfection reaction. At 8 h after transfection, 80 hemagglutinin units of Sendai virus were added to transfected cells. Twenty-four hours after transfection, the luciferase assay was performed with Promega's Dual-luciferase assay kit. Each sample was duplicated and each experiment was repeated at least three times.

**Reverse Transcription-Coupled–Polymerase Chain Reaction (RT-PCR).** 293T cells grown in 60-mm dishes were transfected with pCR3.1-IRF7A (0.4 $\mu$g) alone or together with pCR3.1-ORF45 (1.6 $\mu$g). Sixteen hours after transfection, 80 hemagglutinin units of Sendai virus was added to the transfected cells or cells were left uninfected as a control. Six hours after infection, total RNA was isolated by using Trizol reagent (Life Technologies). The RNA samples were treated with DNase I at 37°C for 30 min. RT-PCR was performed by standard protocol. The primers used in this experiment are as follows. IFNA (consensus primer for all IFN-$\alpha$ subtypes) sense, 5′-AGA ATC TCT CCT TTC TCC TG-3′; IFNA antisense, 5′-TCT GAC AAC CTC CCA GGC AC-3′; IFNA1 sense, 5′-GCA ATA TCT ACG ATG GCC TC-3′; IFNA1 antisense, 5′-CAG AAT TTG TCT AGG AGG TC-3′; $\beta$-actin sense, 5′-ACA ATG AGC TGC TGG TGG CT-3′; $\beta$-actin antisense, 5′-GAT GGG CAC AGT GTG GGT GA-3′. To estimate relative amounts of mRNAs, PCRs were performed on serially diluted samples of reverse transcription products as indicated.

## Results

**KSHV ORF45 Binds to Cellular IRF-7 in Yeast and *in Vitro*.** ORF45 is an immediate-early protein of KSHV (26). The function of this viral protein was unknown. To explore the function of ORF45, we attempted to search for cellular proteins that interact with it in hope that we could get a clue about the function of ORF45 by knowing the protein(s) being associated with it. To this end, we screened a human lymphocyte cDNA library by using the *Saccharomyces cerevisiae* two-hybrid system. The full-length ORF45 coding sequence was cloned into the pAS2–1 vector. This construct expressed a fusion protein with the DNA-binding domain of yeast GAL4 protein and KSHV ORF45 protein that served as the "bait." One million transformants were screened on $Leu^-$ $Trp^-$ $His^-$ plus 3-AT (3-amino-1,2,4-triazole) plates. Twenty $His^+$ $LacZ^+$ colonies were obtained. The prey plasmids from each of these clones were recovered and sequenced. Four clones were found to contain truncated cDNAs of IRF-7. To confirm the interaction between IRF-7 and ORF45, these IRF-7 plasmids were used to retransform yeast cells. $\beta$-Galactosidase (LacZ) activity was detected only when both bait and prey plasmids were present. Then, the interaction of IRF-7 and ORF45 was examined *in vitro*. IRF-7 sequence was subcloned into a pGEX-5X-2 vector, and GST-IRF-7 fusion protein was synthesized in *Escherichia coli* and used to generate an affinity matrix. ORF45 proteins were translated *in vitro* in the presence of [$^{35}$S]methionine and mixed with GST (Fig. 1*A*, lane 3) or GST-IRF-7 immobilized on glutathione-agarose (lane 5). Complexes were washed and resolved by PAGE. As shown in Fig. 1*A*, ORF45 can bind strongly and specifically to GST-IRF-7 but not to GST alone. As a control, luciferase protein can bind neither GST (lane 4) nor GST-IRF7 (lane 6).

To further characterize the interaction between ORF45 and IRF-7, we attempted to map the region(s) of ORF45 that are required for interaction with IRF-7. A series of deletion mutants

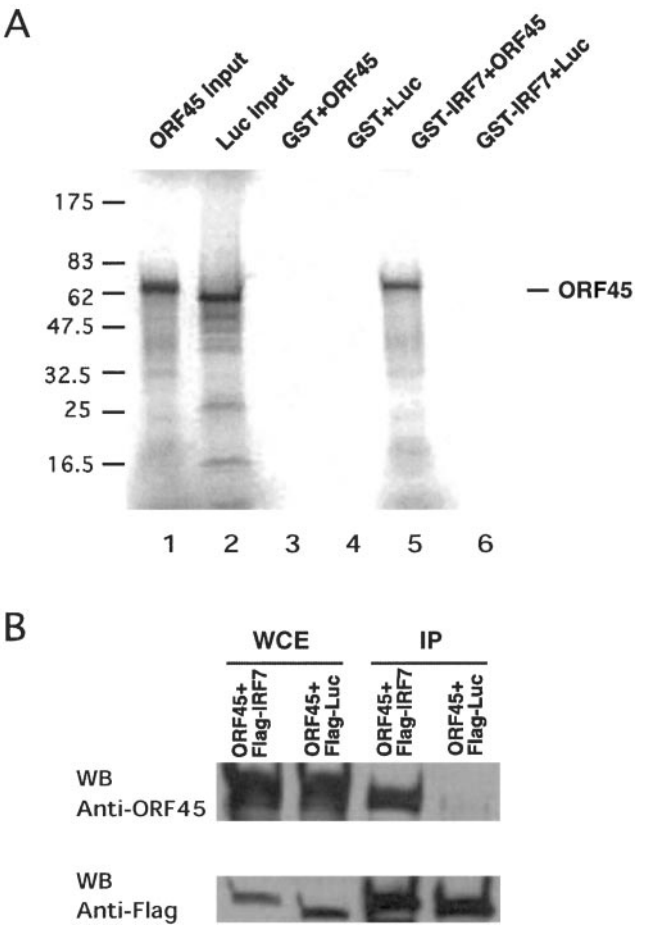


**Fig. 1.** Interaction between KSHV ORF45 and cellular IRF-7 proteins. (*A*) *In vitro* transcribed and translated ORF45 or luciferase (Luc) proteins were incubated with immobilized GST or GST-IRF-7 fusion protein. Bound proteins were resolved on SDS/PAGE. Positions of molecular mass standards (kDa) are shown on the left. (*B*) ORF45 binds to IRF-7 *in vivo*. 293T cells were cotransfected with ORF45- and Flag-IRF-7-expressing vectors. After 24 h, whole cell extracts (WCE) were prepared and subjected to immunoprecipitation (IP) with Flag affinity resin followed by Western blotting (WB) with anti-ORF45 or anti-Flag antibodies.

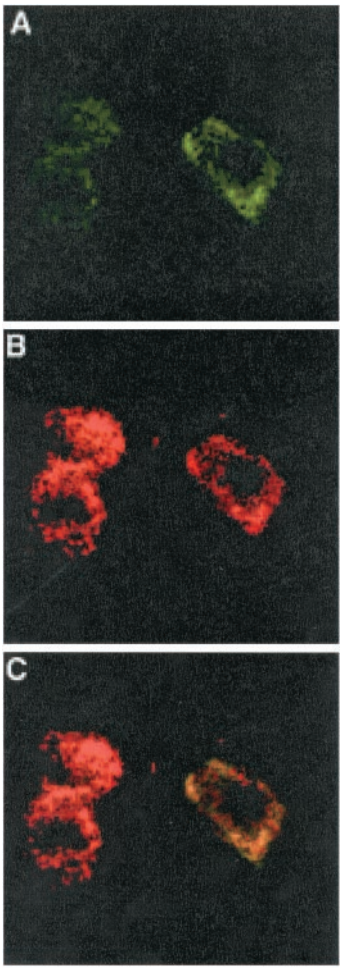


**Fig. 2.** Colocalization of ORF45 and Flag-tagged IRF-7 in 293T cells. Confocal micrographs present 293T cells transfected by plasmids expressing Flag-IRF-7 and ORF45 and stained with mouse monoclonal anti-Flag (*A*) and rabbit polyclonal anti-ORF45 antibodies (*B*). Merged image is presented in *C*.

of ORF45 were constructed and assayed for their abilities to bind GST-IRF-7 fusion protein in an *in vitro* pull-down experiment. The binding affinity of each mutant was determined by the ratio of amount of ORF45 derivative bound to IRF-7 beads versus total input protein. The relative binding abilities of these deletion mutants in comparison to that of wild-type ORF45 are shown in Fig. 7, which is published as supporting information on the PNAS web site, www.pnas.org. It appears that both the N and C termini are involved in the binding. The 17 amino acid residues from the N terminus and the 169 amino acid residues near the C terminus contribute most to the interaction with IRF-7. However, we cannot rule out the possibility that the loss of binding ability of these N- or C-terminal deletions to IRF-7 could be attributed to lack of the correct conformation of these mutants.

**ORF45 Colocalizes and Physically Associates with IRF-7 in Cells.** To confirm that the interaction of ORF45 and IRF-7 indeed occurs *in vivo*, ORF45 and Flag-tagged IRF-7 expression vectors (pCR3.1-ORF45 and pCMV-tag2c-IRF7A, respectively) were constructed and used to transfect 293T cells. Twenty-four hours after transfection, the cells were lysed and subjected to immunoprecipitation using an anti-Flag antibody-agarose affinity gel. Flag-IRF-7-bound ORF45 was detected by immunoblotting using a polyclonal antibody to ORF45. As shown in Fig. 1*B*, ORF45 can be coprecipitated with Flag-IRF-7, but not with Flag-luciferase. This result demonstrated that ORF45 and IRF-7 are physically associated in a complex in cells.

IRF-7 is localized in the cytoplasm before viral infection. When activated by phosphorylation in response to viral signals, it is transported to the nucleus, where it activates type I IFN genes (12, 13, 27, 28). To examine whether KSHV ORF45 is localized in the same compartment with IRF-7 in cells, expression vectors for ORF45 and Flag-IRF-7 fusion proteins were cotransfected into 293T cells. The transfected cells were costained with rabbit polyclonal anti-ORF45 antibody and mouse monoclonal anti-Flag antibody to detect ORF45 and Flag-tagged IRF-7 fusion proteins. The localization of Flag-tagged IRF-7 and ORF45 were examined by immunofluorescence under a confocal microscope. The result showed that the majority of transfected cells expressed both ORF45 and IRF-7 and both are exclusively localized in the cytoplasm (Fig. 2 *A* and *B*). The staining of ORF45 and IRF-7 almost completely overlapped (Fig. 2*C*), again suggesting that ORF45 and IRF-7 are associated with each other in the cytoplasm of the cells.

**ORF45 Blocks Virus-Mediated Phosphorylation of IRF-7.** Phosphorylation is central to activation of IRF-7 in response to viral

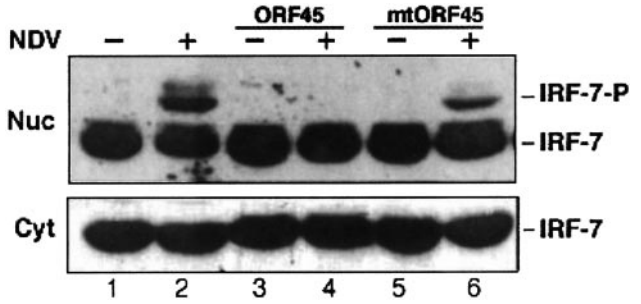


**Fig. 3.** ORF45 inhibits IRF-7 phosphorylation. 293T cells were cotransfected with an IRF-7 expression vector along with ORF45 construct (or mutant ORF45 mt5). Cells were infected with Newcastle disease virus (NDV) at 12 h after transfection and harvested at 7 h after infection. Nuclear and cytoplasmic extracts were prepared and analyzed by Western blotting using an anti-IRF-7 antibody. The mobilities of the basal IRF-7 and phosphorylated IRF-7 (IRF-7-P) are indicated.

infection (27, 28). The phosphorylation leads to homodimerization of IRF-7 and nuclear accumulation of dimers that are competent to bind DNA and transactivate IFN genes (28). To study the effect of ORF45 on function of IRF-7, we asked whether ORF45 had any effect on virus-induced phosphorylation of IRF-7. 293T cells were cotransfected with ORF45 and mouse IRF-7 expression vectors, followed by infection of transfected cells with Newcastle disease virus (NDV). In the absence of ORF45, infection of transfected cells with NDV led to phosphorylation of IRF-7, as judged by the appearance of a mobility-shifted band on Western blot analysis (Fig. 3, lane 2). The phosphorylated form of IRF-7 was absent from the cells in which ORF45 was expressed (lane 4). In contrast, when a deletion mutant of ORF45, namely mt5, which exhibited little ability of binding to IRF-7 *in vitro* (see Fig. 7, published as supporting information on the PNAS web site), was cotransfected with IRF-7 vector into cells, no significant reduction in IRF-7 phosphorylation was observed (Fig. 3, lane 6). This result indicated that ORF45 blocks virus-mediated phosphorylation of IRF-7.

**ORF45 Suppresses Virus-Mediated Nuclear Accumulation of IRF-7.** Because virus-induced phosphorylation is thought to lead to nuclear translocation or retention of IRF-7, we examined the nuclear accumulation of IRF-7 in response to viral infection in the absence and the presence of ORF45. The pCR3.1-ORF45 expression vector or the empty pCR3.1 plasmid was used to cotransfect 293T cells with pEGFP-IRF7A to express ORF45 and GFP-IRF-7 fusion proteins in the cells. Sixteen hours later, the transfected cells were challenged with Sendai virus for 12 h. ORF45 was consistently restricted in the cytoplasm regardless of virus infection (Fig. 4 *A* and *B*). In agreement with Au *et al.* (10), IRF-7 was expressed exclusively in the cytoplasm before viral infection (Fig. 4*C*). In the absence of ORF45, IRF-7 began to accumulate in the nucleus at 4 h after infection. At 12 h, IRF-7 was mainly localized in the nucleus of a majority of the cells (Fig. 4*D*). In the cells that were cotransfected with pCR3.1-ORF45 and pEGFP-IRF7A, GFP-IRF-7 fusion protein failed to be transported from the cytoplasm to the nucleus after being challenged with Sendai virus (Fig. 4*E*).

To confirm the correlation between expression of ORF45 and inhibition of IRF-7 nuclear accumulation, the cells cotransfected with pCR3.1-ORF45 and pEGFP-IRF7A were stained with polyclonal anti-ORF45 antibody. The GFP fluorescence and anti-ORF45 staining were viewed simultaneously under a confocal microscope. The majority of the cotransfected cells expressed both GFP-IRF-7 and ORF45, and GFP-IRF-7 remained in the cytoplasm after the cells were challenged with Sendai virus. A few cells were found not to express ORF45 and in those cells, GFP-IRF-7 accumulated in the nucleus in response to

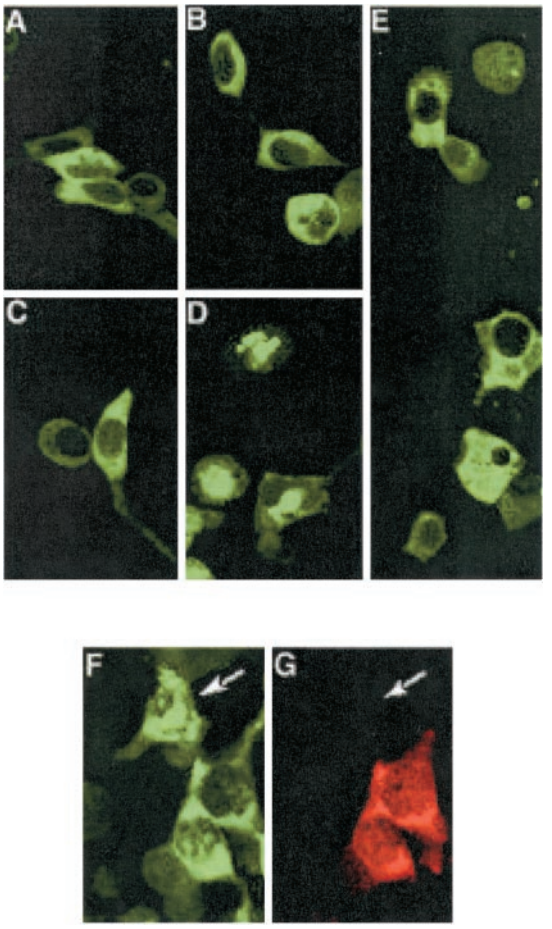


**Fig. 4.** ORF45 suppresses virus-mediated nuclear translocation of IRF-7. 293T cells were transfected with pEGFP-ORF45 and infected with Sendai virus (*B*) or left uninfected (*A*). 293T cells were transfected with pEGFP-IRF7A and infected with Sendai virus (*D*) or left uninfected (*C*). When cells were cotransfected with pEGFP-IRF7A and pCR3.1-ORF45, followed by Sendai virus infection, the nuclear translocation of GFP-IRF-7 was inhibited (*E*). The cells, which were cotransfected with pEGFP-IRF7A and pCR3.1-ORF45 and challenged with Sendai virus, were stained with a rabbit polyclonal anti-ORF45 antibody. Anti-rabbit IgG (Texas red) was used as a secondary antibody. The green fluorescence of GFP-IRF-7 and the red ORF45 staining of the cells are shown in *F* and *G*. Arrows indicate a cell that expresses GFP-IRF-7 but not ORF45.

Sendai viral infection (one such cell is shown in Fig. 4 *F* and *G*). These results suggested that KSHV ORF45 efficiently suppressed the virus-induced nuclear accumulation of IRF-7.

**ORF45 Inhibits Virus-Mediated Induction of Type I IFN Genes.** The critical role of IRF-7 in the induction of type I IFN genes in response to viral infection has been well documented (10–12, 14, 27). We asked whether the blockade of IRF-7 phosphorylation and nuclear accumulation by ORF45 results in inhibition of virus-mediated activation of type I IFN genes. To examine the effect of ORF45 protein on the transcription of IFN-$\alpha$ (*IFNA*) gene, a reporter construct in which a luciferase gene is controlled by human *IFNA1* promoter was cotransfected into 293T cells together with IRF-7 and ORF45 expression plasmids. In the absence of ORF45, the activity of the *IFNA1* promoter in cells was enhanced up to 22-fold by expressing IRF-7. Sendai virus infection further stimulated the promoter by an additional 270-fold. Introduction of the ORF45 expression vector in varied amounts (20–300 ng) resulted in inhibition of *IFNA1* promoter from 38% (20 ng of pCR3.1-ORF45 DNA) to 85% (300 ng of

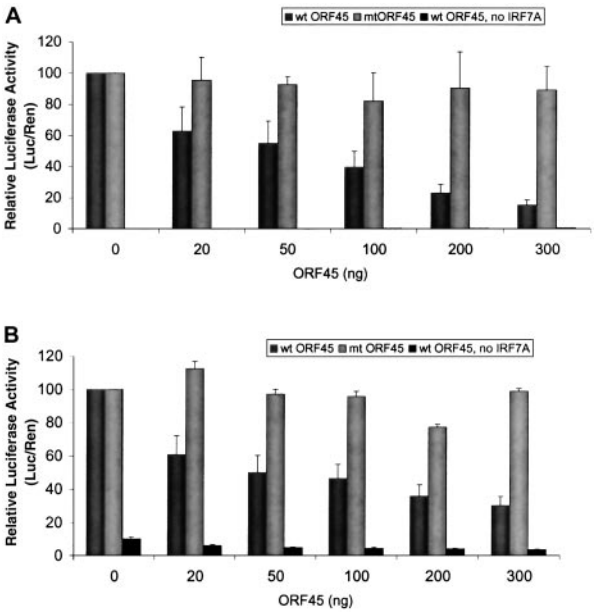


**Fig. 5.** Effect on *IFNA* and *IFNB* promoters of ORF45. Reporter plasmids containing firefly luciferase gene under the control of human *IFNA1* (*A*) and *IFNB* (*B*) promoters were cotransfected with pCR3.1-IRF7A and increasing amounts of pCR3.1-ORF45. At 8 h after transfection, cells were challenged with Sendai virus. At 24 h after transfection, cells were lysed and luciferase activities (Luc) were measured. *Renilla* luciferase (Ren) gene was used as an internal control. Cells cotransfected with a mutant ORF45 (mt5) that shows little binding to IRF-7 and cells expressing no IRF-7 were also assayed for luciferase activities.

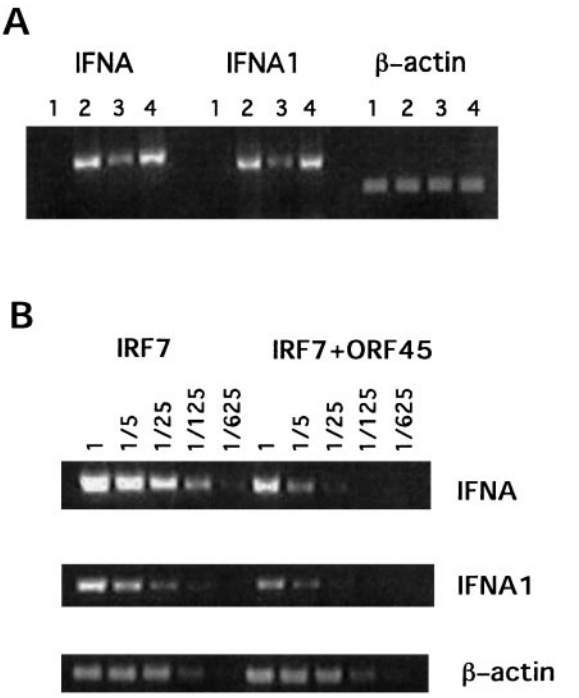


**Fig. 6.** Induction of IFN-$\alpha$ mRNA by Sendai virus is inhibited in 293T cells expressing ORF45. (*A*) IFN-$\alpha$ mRNA levels were analyzed in cells expressing IRF-7 (uninfected) (lanes 1); cells expressing IRF-7 and infected with Sendai virus (lanes 2); cells cotransfected with IRF-7 and ORF45, and infected with Sendai virus (lanes 3); and cells cotransfected with IRF-7 and a truncated ORF45 (mt5), and infected with Sendai virus (lanes 4). RT-PCRs were performed with consensus *IFNA* primers, specific *IFNA1* primers, and $\beta$-actin primers, respectively. (*B*) Relative amounts of total IFN-$\alpha$ (*IFNA*) and IFN-$\alpha$1 (*IFNA1*) mRNAs expressed in cells were compared by serial dilution RT-PCR, as indicated.

DNA) (Fig. 5*A*). To ensure that the effects of ORF45 are specific to *IFNA* transcription and act through binding to IRF-7, several lines of control were included in the experiment. First, in the absence of Sendai virus infection, transfection of 239T cells with IRF-7 resulted in 22- to 47-fold induction of *IFNA1* promoter activity. ORF45 showed little inhibitory effect on the virus-independent *IFNA1* promoter activity (Fig. 8, which is published as supporting information on the PNAS web site). Second, the ORF45 deletion mutant (mt5) was used to cotransfect 293T cells with IRF-7 and IFNA-luciferase reporter vectors. Expression of this truncated ORF45 mutant did not appear to have any significant effect on the activity of *IFNA1* promoter (Fig. 5*A*). Third, an irrelevant promoter, the KSHV ORF59 (DNA processivity factor) promoter, was examined for the effects of IRF-7 and ORF45. ORF59 promoter was induced by KSHV ORF50 (Rta) by 64-fold, but was not activated by IRF-7. Cotransfection with ORF45 showed no synergic effect with either ORF50 or IRF-7 on ORF59 promoter (data not shown). Finally, effects of ORF45 on mutant *IFNA1* promoters in which the positive regulatory domain I (PRD I) and PRD III (13) have been mutated were examined, and the results are shown in Fig. 8, which is published as supporting information.

Because the *IFNB* gene can also be induced by IRF-7 (27, 29), we examined the effect of ORF45 on the human *IFNB* promoter. In the absence of ORF45, transfection of IRF-7, followed by Sendai virus infection, caused 64-fold enhancement of the *IFNB* promoter activity. Expression of ORF45 resulted in inhibition of *IFNB* promoter from 39% (20 ng of DNA) to 70% (300 ng of DNA) (Fig. 5*B*). The truncated ORF45 mutant (mt5) failed to prevent IRF-7 from activating the promoter (Fig. 5*B*). Taking these results together, we conclude that ORF45 inhibits the activation of *IFNA* and *IFNB* promoters by IRF-7 after Sendai virus infection in a dose-dependent manner.

Next, we asked whether ORF45 inhibits virus-mediated induction of *IFNA* genes. 293T cells expressing IRF-7 alone or in combination with ORF45 were challenged with Sendai virus. The induction of IFN-$\alpha$ mRNAs was determined by using RT-PCR. Two sets of primers were used in this assay. One was designed to detect mRNAs of all IFN-$\alpha$ subtypes (especially IFN-$\alpha$1, -2, -4, -7, -10, -13, and -14) and the other to detect only IFN-$\alpha$1 mRNA (the most abundant IFN-$\alpha$ subtype (13, 29, 30). As shown in Fig. 6*A*, induction of *IFNA* gene expression by Sendai virus was significantly reduced in the cells expressing ORF45 (lane 3 vs. lane 2). In contrast, the truncated ORF45 (mt5) did not inhibit the expression of *IFNA* genes (lane 4). The level of the inhibition of *IFNA* gene expression was estimated by using serial dilution RT-PCR (Fig. 6*B*). The level of mRNAs for mixed IFN-$\alpha$ subtypes in cells expressing ORF45 and IRF-7 was about 1/20 of that in the cells expressing IRF-7 only. In addition, the expression of *IFNA1* gene was inhibited by 85% in the cells expressing ORF45 (Fig. 6*B*).

## Discussion

The aim of this study was to explore the function of KSHV immediate-early protein ORF45 and its role in viral infection and tumorigenesis. We found that ORF45 interacts with IRF-7 and inhibits its phosphorylation and nuclear accumulation. IRF-7 serves as direct transducer of virus-mediated signaling from the cytoplasm into the nucleus and plays a critical role in the induction of IFN-$\alpha/\beta$ gene expression. In response to viral infection, IRF-7 is activated through phosphorylation and transported to the nucleus, where it participates in the activation of type I IFN ($\alpha$ and $\beta$) gene transcription (10–12, 29). By blocking phosphorylation and nuclear accumulation of IRF-7, ORF45 efficiently inhibits the activation of type I IFN genes during viral

infection. These results suggest that ORF45 is a protein that KSHV makes and uses to target components of the host antiviral defenses.

The next question, which has not been resolved, is what is the role of ORF45 in the life cycle and pathogenicity of KSHV. Induction of IFN is the earliest immune response against viral infection. It has been shown that IFN-$\alpha$ has an inhibitory effect on KSHV reactivation *in vitro* (31, 32). In clinical practice, IFN-$\alpha$ has been used to treat AIDS-related Kaposi's sarcoma patients (33, 34). These observations indicate that IFN plays important roles in controlling KSHV replication. Thus, a countermeasure used by the virus, presumably provided by ORF45, could be an important virulence factor for the virus. Our preliminary data show that the treatment of BCBL-1 cells, a primary effusion lymphoma cell line that has been latently infected by KSHV, with 500 units/ml IFN-$\alpha$2a gives rise to about 50% inhibition of reactivation upon induction by phorbol 12-tetradecanoate 13-acetate (TPA). Blockade of ORF45 expression by an antisense RNA resulted in a higher degree of inhibition of viral reactivation by IFN-$\alpha$, and the viral reactivation rate upon TPA induction dropped to a level close to that of viral spontaneous reactivation (unpublished data). Although more studies need to be done to precisely determine the functional role of this protein, the trend is clear that ORF45 can rescue KSHV reactivation from the inhibition by IFN-$\alpha$ *in vitro*. Furthermore, the ORF45-involved immune evasion mechanism may also contribute to the reciprocal interaction between KSHV and HIV. Increasing evidence suggests that KSHV and HIV-1 interact to promote Kaposi's sarcoma and AIDS development in individuals who have been dually infected with these two viruses (35–37). Recently, it was reported that among the four KSHV immediate-early proteins we identified, ORF45 (KIE-2) interacted synergistically with HIV-1 Tat in activating expression from the HIV-1 LTR promoter (38). It should be noted that IFN-regulatory factors are multifunctional proteins, involving cell cycle regulation, apoptosis, and tumor suppression or promotion aside from their roles in immune regulation (3–5). Thus, the role of ORF45 through interacting with IRF-7 may not be limited to IFN regulation.

Among the four IRF-7 cDNA clones derived in our yeast two-hybrid screen, the smallest one encodes an IRF-7 fragment at the C terminus between amino acids 255 and 503. Further mapping of IRF-7 for the domain required for interaction with KSHV ORF45 has narrowed it down to 183 aa between residues 283 and 466 (unpublished data). This region contains two functional domains as described by Lin *et al.* (39). The region between amino acids 305 and 467 was found to be an inhibitory domain (ID) in IRF-7. It was shown that deletion of the ID from IRF-7 resulted in constitutive activity in type I IFN gene induction. This mutant activated transcription to a level that was 40-fold higher than seen with wild-type IRF-7 (39). The region between amino acids 247 and 305 contributes to virus inducibility. Deletion of this region abolished the virus inducibility (39). The detailed mechanism underlying the inhibition of IRF-7 nuclear translocation by ORF45 requires further investigation.

Overall, we describe a strategy of viral immune evasion in which KSHV inhibits IFN induction through a viral protein preventing a crucial transcription factor (IRF-7) from being activated. ORF45 appears to be a virulence factor important for KSHV reactivation. Thus, it may serve as a potential target for therapeutic intervention in treatment of KSHV-associated diseases.

We thank Dr. Gary Cohen at the University of Pennsylvania for critical reading of the manuscript. We are grateful to Dr. Charles Whitbeck for help in generation of ORF45 recombinant baculovirus. We thank Dr. Michael Brunda in Roche Pharmaceuticals for providing human IFN-$\alpha$. This work was supported by National Institutes of Health Grants CA86839 (to Y.Y.) and AI46503 (to D.E.L.).

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