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Herpes Simplex Virus 1 Tegument Protein US11 Downmodulates the RLR Signaling Pathway via Direct Interaction with RIG-I and MDA-5

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The interferon (IFN)-mediated antiviral response is a major defense of the host immune system. In order to complete their life cycle, viruses must modulate host IFN-mediated immune responses. Herpes simplex virus 1 (HSV-1) is a large DNA virus containing more than 80 genes, many of which encode proteins that are involved in virus-host interactions and show immune modulatory capabilities. In this study, we demonstrate that the US11 protein, an RNA binding tegument protein of HSV-1, is a novel antagonist of the beta IFN (IFN-β) pathway. US11 significantly inhibited Sendai virus (SeV)-induced IFN-β production, and its double-stranded RNA (dsRNA) binding domain was indispensable for this inhibition activity. Additionally, wild-type HSV-1 coinfection showed stronger inhibition than US11 mutant HSV-1 in SeV-induced IFN-β production. Coimmunoprecipitation analysis demonstrated that the US11 protein in HSV-1-infected cells interacts with endogenous RIG-I and MDA-5 through its C-terminal RNA-binding domain, which was RNA independent. Expression of US11 in both transfected and HSV-1-infected cells interferes with the interaction between MAVS and RIG-I or MDA-5. Finally, US11 dampens SeV-mediated IRF3 activation. Taken together, the combined data indicate that HSV-1 US11 binds to RIG-I and MDA-5 and inhibits their downstream signaling pathway, preventing the production of IFN-β, which may contribute to the pathogenesis of HSV-1 infection.

The innate immune response constitutes the first line of host defense that limits viral spread and also plays an important role in the activation of the adaptive immune response. The innate immune response to viral infection involves the recognition of viral components through pathogen recognition receptors (PRRs) and the subsequent induction of type I interferons (IFNs), including alpha/beta IFN (IFN-α/β), which can trigger the expression of antiviral proteins. Host PRRs recognize pathogen-associated molecular patterns (PAMPs), such as viral nucleic acids (65, 82). PRRs comprise membrane-associated receptors, such as the Toll-like receptors (TLRs), and cytosolic receptors, including RIG-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (85). More recently, several cytosolic DNA sensing receptors have been identified: the DNA-dependent activator of interferon regulatory factors (DAI) (81), absent in melanoma 2 (AIM2) (7, 27), RNA polymerase III (Pol III) (16), leucine-rich repeat (in Flightless I) interacting protein 1 (Lrrfip1) (91), DExD/H box helicases (DHX9 and DHX36) (36), and the IFN-inducible protein IFI16 (84).

The presence of double-stranded RNA (dsRNA), a by-product of viral replication, is recognized as a PAMP by Toll-like receptor 3 (TLR3) and two caspase recruitment domain (CARD)-containing RNA helicases, retinoic acid-inducible gene I (RIG-I) and melanoma-associated differentiation gene 5 (MDA-5), which act as cytoplasmic sensors of dsRNA (1, 28, 66, 80, 92, 93). Whereas TLR3 mainly senses extracellular dsRNA on antigen-presenting cells, RIG-I and MDA-5 are constitutively expressed and detect intracellular dsRNA (1, 93). TLR3 signals through an adaptor called TIR domain-containing adaptor inducing IFN-β (TRIF), while RIG-I and MDA-5 recruit another CARD-containing adaptor, called mitochondrial antiviral signaling protein (MAVS) (also referred to as IPS-1, Cardif, or VISA), to relay signals to the kinases TBK1 and inducible IκB kinase (IKKi), which phosphorylate interferon regulatory factor 3 (IRF3), and to IκB kinase beta (IKKβ), which activates the NF-κB pathway (24, 34, 58, 75, 90). Once activated, IRF3 translocates into the nucleus and binds positive regulatory domains I and III (PRDIII-I) of the IFN-β promoter to induce the expression of IFN-β. In turn, newly synthesized IFN-β, which is considered a hallmark of the antiviral response, induces the expression of interferon-stimulated genes (ISGs), including those encoding proteins such as 2′-5′ oligoadenylate synthase (OAS) and dsRNA-dependent protein kinase R (PKR) and interferon-stimulated genes 15 and 56 (ISG15 and ISG56), which are responsible for the establishment of an antiviral state in infected cells and in neighboring noninfected cells (68, 74, 82).

To establish a persistent infection, viruses have evolved mechanisms to evade the host immune response. A passive form of evasion is that viral gene expression is silenced and the infected cells are therefore invisible to immune responses. In addition, active mechanisms of immune evasion are frequently evident during the productive stage of the virus life cycle. Several human viruses, including hepatitis C virus (42), vaccinia virus (78), Ebola virus (5), and influenza virus (83), have evolved strategies to target and inhibit distinct steps in the early signaling events that lead to type I IFN induction, indicating the importance of type I IFN in the host antiviral response.

Herpes simplex virus 1 (HSV-1) is a large DNA virus known to

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encode several gene products that enable viral evasion of the host innate immune response (41, 54, 61). HSV-1 infection in cell culture induces a host type I IFN response, which is subsequently shut down by HSV-encoded gene products (55, 57). Several studies have shown that HSV-1 encodes ICP0, an immediate-early gene product that can inhibit the transcription factor IRF3 (48, 51, 53, 60). ICP27, another immediate-early gene product, has been shown to antagonize type I IFN signaling (30). The HSV-encoded late gene products γ34.5 and US11 inhibit the antiviral functions of PKR (26, 56, 67, 86). US3, an HSV-encoded kinase, was shown to block the expression of IFN-γ-dependent genes (45). Last, vhs, a tegument protein and HSV-encoded late gene product, degrades both host and viral mRNA (22, 39, 77), and in mouse models it seems to play important roles in HSV-1 pathogenesis (77, 79). Elucidating HSV-1 immune evasion mechanisms at mucosal surfaces during recurrent infection may provide new strategies for antiviral drug and HSV vaccine development.

Despite its association with various human health problems, our knowledge of the HSV-1 evasion strategies against type I IFN-mediated host innate immunity is still not complete. To search for specific HSV genes that block IFN production, we carried out a screen of HSV-1 open reading frames (ORFs) for their abilities to block the IFN-dependent antiviral response. In this study, we demonstrate that US11, an RNA binding tegument protein of HSV-1, is a novel antagonist of IFN-β production. We provide evidence that US11 binds to endogenous RIG-I and MDA-5 and inhibits downstream activation of the RLR signaling pathway, preventing the transcriptional induction of IFN-β. Collectively, these results suggest that control of RIG-I and MDA-5 by US11 impairs type I IFN responses, which may contribute to the pathogenesis of HSV-1 infection.

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MATERIALS AND METHODS

Cells, viruses, and antibodies. HEK 293T cells and Vero cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin and streptomycin. HeLa cells were maintained in Eagle's minimum essential medium (MEM) (Gibco-BRL) supplemented with 10% FBS.

The HSV-1 F strain US11 mutant, which is lacking US11 expression, was kindly provided by Bernard Roizman (72). The wild-type (WT) HSV-1 F strain and US11 mutant HSV-1 were propagated in Vero cells and titrated as described previously (88). Sendai virus (SeV) was propagated in 10-day-old embryonated eggs, and the virus titer was determined by hemagglutination assay using chicken red blood cells.

Rabbit antisera against IRF3-S396 were described previously (18). The protease inhibitor mixture cocktail, mouse anti-Myc (isotype IgG1), and anti-Flag (isotype IgG2b) monoclonal antibodies (MAbs) were purchased from CST (Boston, MA). Mouse anti-hemagglutinin (HA) MAb (isotype IgG2b) was purchased from Roche (Mannheim, Germany). Mouse monoclonal IgG1 and IgG2b isotype control antibodies were purchased from eBioscience Inc. (San Diego, CA). Rabbit anti-IRF3 polyclonal antibody (pAb), rabbit anti-yellow fluorescent protein (YFP) pAb, and mouse anti-β-actin MAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-RIG-I pAb and rabbit anti-MDA-5 pAb were purchased from GeneTex (San Antonio, TX). Rabbit anti-US11 pAb and rabbit anti-VP22 pAb were developed as described in our previous studies (43, 46, 62).

Plasmid construction. All enzymes used for cloning procedures were purchased from Takara (Dalian, China) except T4 DNA ligase (New England BioLabs, Massachusetts). To construct US11-HA, the US11 gene was amplified from plasmid US11-EYFP as described in our previous study (89) and cloned into the BglII and EcoRI sites of the pCMV-HA vector (Beyotime, Shanghai, China). Commercial reporter plasmids include NF-κB-Luc (Stratagene, La Jolla, CA) and pRL-TK plasmid (Promega). Gift plasmids include the following: (PRDIII-I)4-Luc (21), pcDNA3.1-FlagTBK1 and pcDNA3.1/Zeo-MAVS (64), pcDNA3.1-FlagIKKi (94), pEF-Flag-RIG-I, pEF-Flag-MDA-5, pEF-Flag-RIG-IN, and pEF-Flag-RIG-IC (93), pEF-Flag-MDA-5C (the MDA-5 CARD domain, amino acids [aa] 1 to 287) and pEF-Flag-MDA-5H (the MDA-5 helicase domain, aa 287 to 1025) (15), IRF-3/5D (11), pCAGGS-NS1 (37), and pMyc-MAVS and IFN-β promoter reporter plasmid p125-luc (47).

RNA isolation, semiquantitative RT-PCR, and quantification of gel image. Total RNA was extracted from HEK 293T cells with TRIzol (Invitrogen, California) according to the manufacturer's manual. Samples were digested with DNase I and subjected to reverse transcription-PCR (RT-PCR) as previously described (96). RNA was reverse transcribed using an oligo(dT) primer. A mock reaction was carried out with no reverse transcriptase added. Ten percent of the resulting cDNA was used as a template for PCR using primers specific for human IFN-β. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping gene to establish a baseline against which target genes were compared between samples. Primer sequences were as follows: 5′-GACACCCACTCCTCCACCTTT-3′ (forward) and 5′-ACCACCCTGTTGCTGTAGCC-3′ (reverse) for GAPDH and 5′-CAAATTGCTCTCCTGTTGTGCTTC-3′ (forward) and 5′-AATGCGGCGTCCTCCTTCT-3′ (reverse) for IFN-β. PCR products were analyzed on a 2% agarose gel. All analyses were based on data from one representative of at least three experiments.

Transfection and dual-luciferase reporter (DLR) assay. HEK 293T cells were plated on 24-well dishes (Corning, NY) in DMEM (Gibco-BRL, Maryland) with 10% FBS at a density of 1×10^5 cells per well overnight before transfection as previously described (96). Cells were then cotransfected with 500 ng reporter plasmid, such as p125-luc, NF-κB-Luc, or (PRDIII-I)4-Luc, and 1 μg expression plasmid, as indicated by standard calcium phosphate precipitation (32, 95). To normalize transfection efficiency, 50 ng of pRL-TK *Renilla* luciferase reporter plasmid was added to each transfection. At 24 h posttransfection, cells were infected with 100 hemagglutination units (HAU) SeV ml^{-1} for 16 h, and then luciferase assays were performed with a dual-specific luciferase assay kit (Promega, Madison, WI) as previously described (96). All reporter assays were completed at least in triplicate, and the results were shown as average values ± standard deviations (SD) from one representative experiment.

Coimmunoprecipitation assay. Coimmunoprecipitation (co-IP) assays were performed as described in our previous study (88). Briefly, HEK 293T cells ($\sim 5 \times 10^6$) were cotransfected with 10 μg of each of the indicated expression plasmids carrying FLAG, Myc, or HA tags. Transfected cells were harvested at 24 h posttransfection and lysed on ice with 1 ml of lysis buffer. For each immunoprecipitation (IP), a 0.5-ml aliquot of lysate was incubated with 0.5 μg of the anti-HA MAb, anti-Myc MAb, or anti-Flag MAb or nonspecific mouse monoclonal antibody (IgG1 isotype matched with anti-Myc MAb; IgG2b isotype matched with anti-HA and anti-Flag MAbs) and 30 μl of a 1:1 slurry of Protein A/G Plus-agarose (Santa Cruz, California) for at least 4 h or overnight at 4°C. The beads were washed four times with 1 ml of lysis buffer containing 500 mM NaCl and then subjected to Western blot (WB) analysis. All co-IP assays were repeated at least two times, and similar data were obtained.

Native PAGE. Native PAGE) was carried out using ReadyGels (7.5%; Bio-Rad). The gel was prerun with 25 mM Tris and 192 mM glycine, pH 8.4, with 1% deoxycholate (DOC) in the cathode chamber for 30 min at 40 mA. Samples in native sample buffer (10 μg protein, 62.5 mM Tris-Cl, pH 6.8, 15% glycerol, and 1% DOC) were size fractionated by electrophoresis for 60 min at 25 mA and transferred to nitrocellulose membranes for WB analysis as described previously (29).

Western blot analysis. Western blot analysis was performed as described in our previous study (88). Briefly, whole-cell extracts were sub-

jected to 12% SDS-PAGE and transferred to nitrocellulose membranes, followed by blocking with 5% nonfat milk in Tris-buffered saline–Tween (TBST) (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.1% [vol/vol] Tween 20) and probed with the appropriate primary antibodies at 37°C for 2 h. After washing with TBST, the membrane was incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG or goat anti-mouse IgG at 37°C for 1 h. To avoid detection of IgG light chains that comigrate with US11-HA in co-IP assays, alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (γ heavy chain specific) (Vicgene Biotechnology, California) was applied. Protein bands specific to the antibody were developed by 5-bromo-4-chloro-3-indolylphosphate (BCIP)–nitroblue tetrazolium (NBT) and terminated by distilled water.

Immunofluorescence assay. Immunofluorescence assays were performed as described previously (89). In brief, HeLa cells were either observed live or fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.137 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4, 0.001 M NaH_2PO_4, pH 7.4) for 20 min, washed three times with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. The cells were rinsed with PBS and then incubated with PBS containing 5% bovine serum albumin (BSA) for 20 min at room temperature. Subsequently, the cells were incubated with rabbit anti-IRF3 pAb (diluted 1:500) or with mouse anti-HA MAb (diluted 1:2,000) for 2 h at 37°C, followed by incubation with tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-rabbit IgG (Pierce) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich) in PBS containing 0.5% BSA for 1 h at 37°C. After each incubation step, cells were washed extensively with PBS. Samples were analyzed using fluorescence microscopy (Zeiss, Germany).

ELISA for IFN-β. An ELISA to quantify secreted IFN-β was carried out with culture supernatants collected from infected cells. Medium was collected and centrifuged to remove cell debris. Fifty microliters of cleared supernatants or the IFN-β standard was used in duplicate for detection of IFN-β using a human IFN-β ELISA kit (PBL InterferonSource, Piscataway, NJ), following the manufacturer's instructions.

RESULTS

US11 inhibits SeV-mediated production of IFN-β. To determine the ability of US11 to inhibit SeV-mediated activation of IFN-β gene transcription, an HA-tagged US11 expression plasmid was cotransfected into HEK 293T cells together with an IFN-β promoter construct. SeV infection resulted in strong induction of IFN-β reporter activity (Fig. 1A). In contrast, ectopic expression of US11 significantly inhibited SeV-mediated activation of IFN-β promoter activity, similar to results for the positive-control influenza A virus NS1 protein (Fig. 1A). Additionally, US11 inhibited IFN-β promoter activity in a dose-dependent manner (Fig. 1B); the expression of the US11 protein was confirmed by Western blot analysis (Fig. 1B).

The activation of IFN-β gene transcription depends on synergistic interactions among NF-κB, IRFs, and other transcription factors that bind to distinct regulatory domains in the promoter. To examine the role of US11 in inhibition of SeV-mediated activation of IRFs and NF-κB, we measured expression of the luciferase reporter gene driven by tandem IRF binding sites from the IFN-β promoter [(pRDIII-I)4-Luc] or by NF-κB elements (NF-κB-Luc). SeV infection resulted in strong induction of NF-κB-Luc and (pRDIII-I)4-Luc reporter activity (Fig. 1C and D). However, coexpression of US11 could significantly inhibit NF-κB-Luc activity (Fig. 1C). Similarly, the activation of the (pRDIII-I)4 reporter gene construct, which depends on the function of IRFs, was significantly decreased with US11 coexpression (Fig. 1D). To further determine the role of the US11 protein in the inhibition of SeV-induced IFN-β production, both IFN-β mRNA accumulation and protein secretion were measured by RT-PCR and ELISA, respectively. As expected, mRNA and protein levels of endogenous IFN-β were strongly upregulated by SeV infection (Fig. 1E and F), whereas US11 significantly reduced the accumulation of endogenous IFN-β mRNA and IFN-β secretion (Fig. 1E and F, respectively). Taken together, these results demonstrate that US11 inhibits SeV-mediated production of IFN-β by blocking IRF and NF-κB activation.

WT HSV-1 shows stronger inhibition than US11 mutant HSV-1 in SeV-induced IFN-β production. To further examine the effect of US11 on induction of IFN-β in HSV-1 infection, both wild-type (WT) HSV-1 and a US11 mutant of HSV-1 (which lacks US11 expression) (72) were employed to test their abilities to inhibit IFN-β production induced by SeV by DLR assays. SeV infection stimulated the promoter activity of IFN-β, but neither WT HSV-1 infection nor US11 mutant HSV-1 infection (multiplicity of infection [MOI] of 1) alone stimulated the promoter activity of IFN-β (Fig. 2A). However, WT HSV-1 infection inhibited SeV-mediated IFN-β promoter activity about 10-fold (Fig. 2A), indicating that one or more proteins expressed during HSV-1 infection were responsible for blocking SeV-induced promoter activity of IFN-β. In contrast, US11 mutant HSV-1 infection inhibited SeV-mediated IFN-β promoter activity about 5-fold (Fig. 2A), and the difference between WT HSV-1 and US11 mutant HSV-1 was statistically significant (Fig. 2A, $P < 0.031$), suggesting that US11 expression during HSV-1 infection might contribute to dampening SeV-induced promoter activity of IFN-β. VP22 expression following WT and US11 mutant HSV-1 infection was detected at the expected molecular mass, whereas US11 expression was detected only following infection with WT HSV-1 (Fig. 2A). Furthermore, semiquantitative RT-PCR and ELISA were performed to measure whether HSV-1 infection affected the mRNA level and protein secretion, respectively, of endogenous IFN-β. As expected, coinfection of US11 mutant HSV-1 showed less inhibition in the SeV-induced mRNA level and protein secretion of endogenous IFN-β than that of WT HSV-1 (Fig. 2B and C), and the difference between WT HSV-1 and US11 mutant HSV-1 was statistically significant (Fig. 2C) ($P < 0.025$), demonstrating that US11 expression during HSV-1 infection contributes to the inhibition of IFN-β production. We concluded from these data that HSV-1 infection blocked SeV-induced IFN-β production and US11 was one of the proteins expressed after HSV-1 infection for inhibiting SeV-induced IFN-β production.

The C-terminal dsRNA binding domain of US11 is responsible for inhibiting SeV-mediated IFN-β promoter activity. US11 is a multifunctional protein of HSV-1 that can be divided into two separable domains (Fig. 3A). The amino terminus is reported to contain a transactivation domain similar to that encoded by complex retroviruses (19), while the carboxyl terminus can bind RNA (70) and contributes to nucleolar localization (10, 70, 89). To determine which region of US11 was responsible for the inhibition of virus-mediated activation of the IFN-β promoter, a series of US11 WT and recombinant plasmids constructed previously (89), including US11-EYFP, US11(1-83)-EYFP, US11(84-152)-EYFP, US11(84-125)-EYFP, and US11(126-152)-EYFP, were employed in this study. SeV infection significantly activated the IFN-β promoter; however, overexpression of full-length US11-EYFP blocked the activation of IFN-β promoter activity, consistent with US11-HA data (Fig. 3B and 1A), indicating that the presence of EYFP in the US11-EYFP fusion protein did not affect

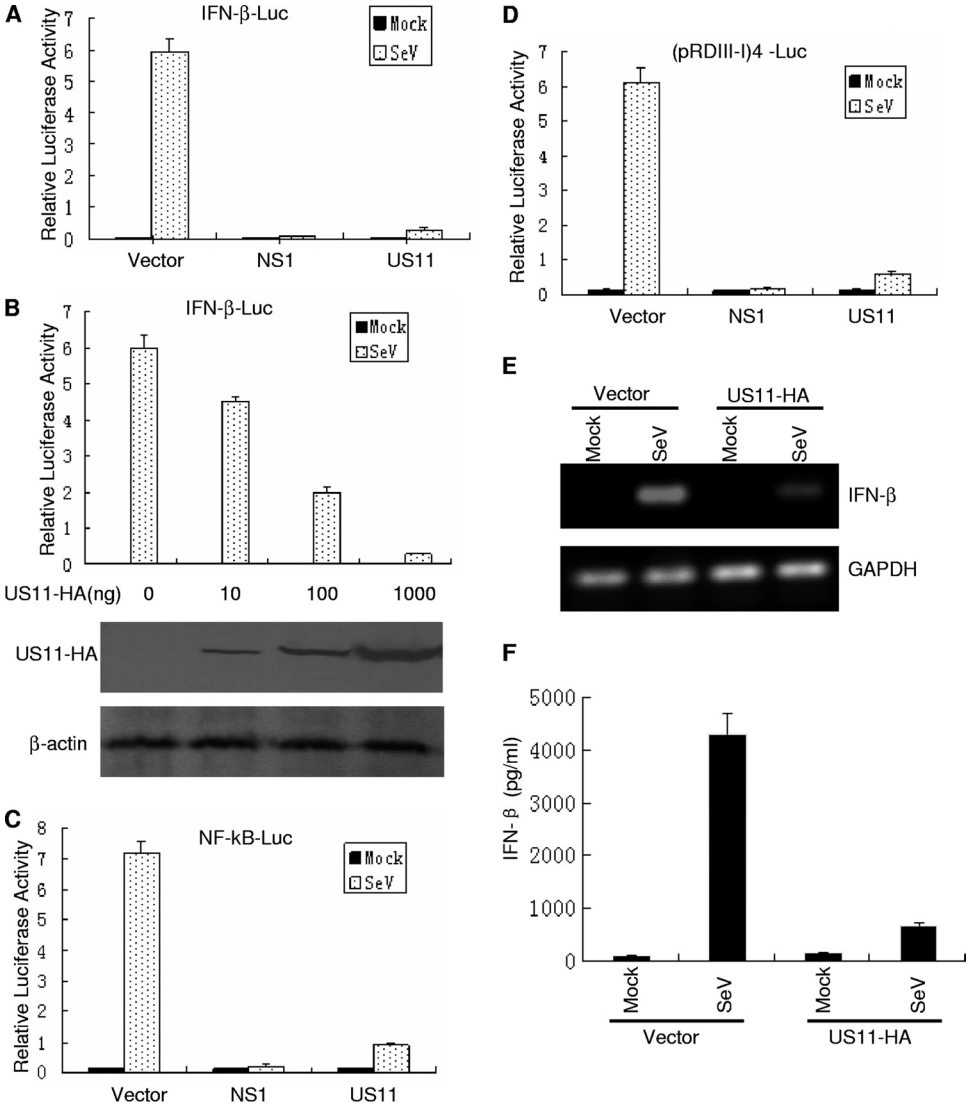

FIG 1 US11 inhibits SeV-mediated activation of the IFN-β and NF-κB promoter activities. (A, C, and D) HEK 293T cells were transfected with 500 ng of IFN-β promoter reporter plasmid p125-luc (A), NF-κB-Luc (C), or (pRDIII-I)4-Luc (D), together with *Renilla* luciferase plasmid pRL-TK (50 ng) and pCMV-HA empty vector or plasmids encoding the indicated viral proteins (1,000 ng). At 24 h after transfection, cells were left untreated or infected with 100 HAU ml^{-1} SeV as indicated, and luciferase activity was measured 16 h postinfection. (B) As in panel A, except an increased amount of US11-HA expression plasmid, as indicated, was used. The expression of US11 was analyzed by Western blotting using anti-HA and anti-β-actin (as a control) monoclonal antibodies. Data are expressed as relative luciferase activities with standard deviations for three independent experiments performed in duplicate. (E) HEK293T cells were transfected with pCMV-HA empty vector or US11-HA expression plasmid. At 24 h posttransfection, cells were mock infected or infected with 100 HAU ml^{-1} SeV for 16 h before RT-PCR was performed using GAPDH and IFN-β primers. (F) Medium from infected cells in panel E was isolated and analyzed by ELISA for IFN-β secretion as described in Materials and Methods. The data represent means + standard deviations for three replicates.

the inhibitory activity of US11. The expression of US11(84-152)-EYFP, containing the dsRNA binding domain, blocked SeV-mediated activation, while the expression of US11(1-83)-EYFP, containing the N-terminal transactivation region, was unable to inhibit SeV-mediated activation of the IFN-β promoter (Fig. 3B). Additionally, the expression of US11(84-125)-EYFP or US11 (126-152)-EYFP only partially inhibited SeV-mediated activation of the IFN-β promoter (Fig. 3B). The expression of the US11-EYFP fusion proteins from the recombinant plasmids was detected at their expected molecular masses (Fig. 3C). These results suggest that the C-terminal dsRNA-binding domain of US11 is responsible for inhibiting SeV-mediated IFN-β activity.

US11 interacts with endogenous RIG-I and MDA-5. It is well established that in response to virus infection, the cytosolic helicases RIG-I and MDA-5 initiate antiviral signaling, whereby a number of downstream molecules are recruited or activated (33). Additionally, both RIG-I and MDA-5 have been found to play a role in IFN production in response to HSV (69). We next determined the molecular mechanism by which US11 inhibits SeV-induced IFN-β production. US11 could not inhibit IFN-β promoter activity induced by overexpression of the N-terminal CARD domain of RIG-I or its downstream signaling molecules, including MAVS, TBK1, IKKi, and IRF3/5D (data not shown), indicating that US11 may block the IFN-β pathway at the RIG-I or

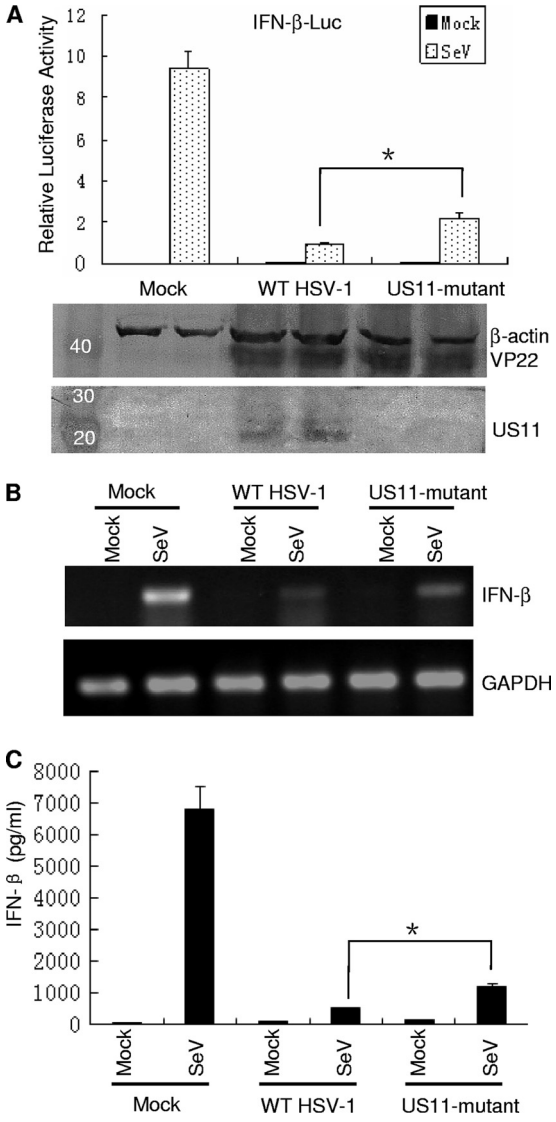

FIG 2 Effects of WT or US11 mutant HSV-1 infection on SeV-induced IFN-β production. (A) HEK 293T cells in a 24-cell plate were cotransfected with the IFN-β reporter plasmid p125-luc and *Renilla* luciferase plasmid pRL-TK and were mock infected or infected with 100 HAU/ml SeV as indicated at 24 h after transfection. At 8 h postinfection, the HEK 293T cells were then mock infected or infected with either WT or US11 mutant HSV-1 at an MOI of 1 for another 16 h until the luciferase activity was measured. Data are expressed as relative luciferase activities with standard deviations for three independent experiments performed in duplicate. Additionally, the expression of both the US11 and VP22 proteins after WT or US11 mutant HSV-1 infection was verified by Western blotting using rabbit anti-US11 or anti-VP22 pAbs. β-Actin as a loading control was also detected. Statistical analysis was performed using Student's *t* test. *, $P < 0.031$. (B) HEK 293T cells were seeded onto a 35-mm cell culture plate after 24 h and were infected with SeV or HSV-1 as described for panel A. Then, total RNA was extracted at indicated time points for semiquantitative RT-PCR analysis as described for Fig. 1E. (C) HEK 293T cells in a 24-cell plate were mock infected or infected with 100 HAU/ml SeV. At 8 h postinfection, the cells were then mock infected or infected with either WT or US11 mutant HSV-1 at an MOI of 1 for another 16 h. Medium from the same infected cells was isolated and analyzed by ELISA for IFN-β secretion as described in Materials and Methods. "Mock" cells were treated with medium without SeV and HSV-1 infection. The data represent means plus standard deviations for three replicates. Statistical analysis was performed using Student's *t* test. *, $P < 0.025$.

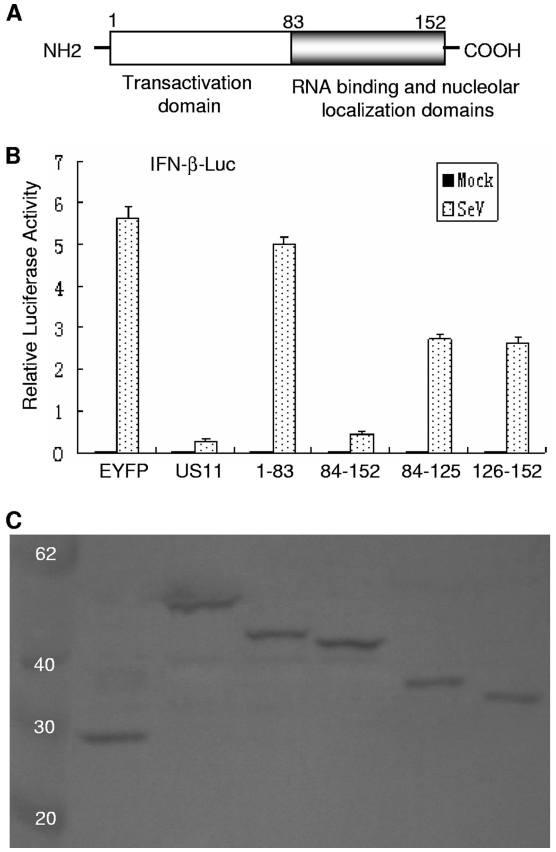

FIG 3 The C-termimal dsRNA binding domain of US11 is responsible for inhibiting SeV-mediated IFN-β promoter activity. (A) Schematic representation of US11 functional domains and deletions. (B) Luciferase assays in HEK 293T cells were performed as for Fig. 1A to measure the activation of the IFN-β promoter following SeV infection in the presence of full-length and deletion mutants of US11, including US11-EYFP, US11(1--83)-EYFP, US11(84-152)-EYFP, US11(84-125)-EYFP, and US11(126-152)-EYFP. Data are expressed as relative luciferase activities with standard deviations for three independent experiments performed in duplicate. (C) The expression of these US11 deletion mutants was verified by Western blotting using rabbit anti-YFP polyclonal antibody.

MDA-5 level. Thus, we investigated the interaction between US11 and RIG-I or MDA-5. US11-HA and either Flag-RIG-I or Flag-MDA-5 expression plasmids were cotransfected into 293T cells, and co-IP/WB analysis was performed with anti-HA and anti-Flag MAbs. Both RIG-I and MDA-5 were efficiently coimmunoprecipitated with US11 by anti-HA MAb (Fig. 4A and 5B) but not by nonspecific mouse monoclonal antibody IgG2b (Fig. 4A and 5B). Since RIG-I, MDA-5, and US11 are RNA binding proteins, positive results from co-IP assays may not reflect direct interactions but may rather suggest a tertiary complex with RNA. To address this possibility, lysates from cells cotransfected with US11 and RIG-I or MDA-5 plasmids were treated with RNase A prior to immunoprecipitation. Both RIG-I and MDA-5 were comparably co-IPed with US11 under RNase A treatment (Fig. 4C and D), indicating that the interaction between US11 and RIG-I or MDA-5 was RNA independent. The goal of the subsequent experiment was to determine whether US11 interacts with RIG-I or MDA-5 in the context of a viral infection. HEK293T cells were transfected with Flag-RIG-I or Flag-MDA-5 and then infected

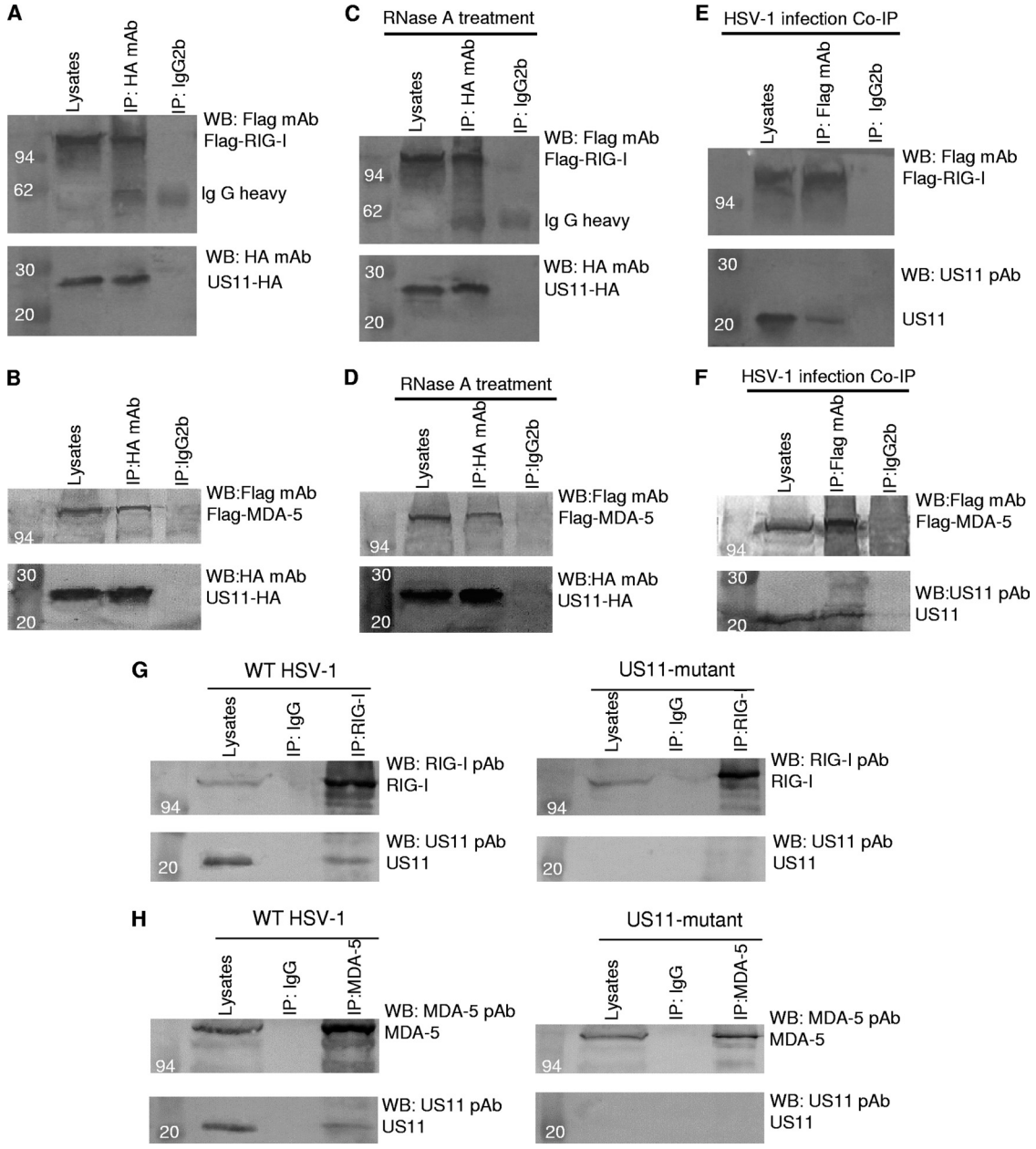

FIG 4 US11 interacts with both RIG-I and MDA-5 in transfected and HSV-1-infected cells. (A, B, C, and D) US11 associates with both RIG-I and MDA-5 in transfected cells. HEK293T cells (~5×10^6) were cotransfected with 10 μg of plasmid US11-HA and with 10 μg of plasmid pEF-Flag-RIG-I, encoding full-length RIG-I (A and C), or with plasmid pEF-Flag-MDA-5, encoding full-length MDA-5 (B and D), respectively. At 36 h after transfection, cells were lysed and clarified supernatants were left untreated (A and B) or treated with RNase A at 150 μg/ml (C and D). The samples were then subjected to immunoprecipitation assays using anti-HA MAb (IP: HA) or nonspecific mouse monoclonal antibody (IgG2b). Cell lysates and immunoprecipitated proteins were separated in denaturing 12% polyacrylamide gels and transferred to nitrocellulose membranes. The transferred proteins were probed with anti-HA and anti-Flag MAbs. (E and F) US11 interacts with overexpressed RIG-I and MDA-5 in HSV-1-infected cells. HEK293T cells were transfected with pEF-Flag-RIG-I (E) or pEF-Flag-MDA-5 (F), respectively. At 20 h after transfection, cells were infected with HSV-1 strain F at an MOI of 10 for 16 h. The cells were then lysed, and the extracts were subjected to immunoprecipitation using anti-Flag MAb (IP: Flag) or nonspecific mouse monoclonal antibody (IgG2b). Precipitates were analyzed by Western blotting using anti-Flag MAb or rabbit anti-US11 pAb. (G and H) US11 interacts with endogenous RIG-I and MDA-5 in HSV-1-infected cells. HEK293T cells were infected with WT or US11 mutant HSV-1 at an MOI of 10 for 16 h. The cells were then lysed, and the extracts were subjected to immunoprecipitation using anti-RIG-I pAb (IP: RIG-I), anti-MDA-5 pAb (IP: MDA-5), or control IgG. Precipitates were analyzed by Western blotting.

with WT HSV-1. US11 could be immunoprecipitated by RIG-I or MDA-5 using anti-Flag MAb but not by nonspecific mouse monoclonal antibody IgG2b (Fig. 4E and F), demonstrating that US11 interacts with overexpressed RIG-I and MDA-5 in the context of viral infection. To determine whether US11 interacts with RIG-I and MDA-5 under physiological conditions, we performed co-IP experiments using endogenous proteins. In WT HSV-1-infected cells, the US11 protein was easily immunoprecipitated by RIG-I or MDA-5 using anti-RIG-I or anti-MDA-5 pAbs (Fig. 4G and H) but not by control antibody IgG (Fig. 4G and H). As

expected, in US11 mutant HSV-1-infected cells, no US11 protein was immunoprecipitated by either RIG-I or MDA-5 (Fig. 4G and H). Collectively, these data suggested that the US11 protein interacts with both endogenous RIG-I and MDA-5 during HSV-1 infection in an RNA-independent manner.

The carboxyl terminus of US11 interacts with the carboxyl terminus of RIG-I and MDA-5. To further determine which region of RIG-I or MDA-5 binds to US11, RIG-I mutants containing either the amino-terminal domain (aa 1 to 229; Flag-RIG-IN) or the carboxyl-terminal domain (aa 218 to 925; Flag-RIG-IC) (93) and MDA-5 mutants containing either the amino-terminal CARD domain (aa 1 to 287; Flag-MDA-5C) or the carboxyl-terminal helicase domain (aa 287 to 1025; Flag-MDA-5H) (15) were tested. In 293T cells cotransfected with US11-HA and either Flag-RIG-IN or Flag-RIG-IC, US11 coimmunoprecipitated with RIG-IC (Fig. 5A) but not with RIG-IN (Fig. 5B). Similarly, in 293T cells cotransfected with US11-HA and either Flag-MDA-5C or Flag-MDA-5H, US11 coimmunoprecipitated with MDA-5H (Fig. 5C) but not with MDA-5C (Fig. 5D). Additionally, US11(84-152)-EYFP was successfully immunoprecipitated by anti-Flag MAb from cells expressing US11(84-152)-EYFP and either Flag-RIG-IC (Fig. 5E) or Flag-MDA-5H (Fig. 5F), while no detectable EYFP was coimmunoprecipitated by anti-Flag MAb from cells expressing EYFP and either Flag-RIG-IC (Fig. 5E) or Flag-MDA-5H (Fig. 5F), further demonstrating that the C-terminal amino acids 84 to 152 of the US11 protein were responsible for interacting with RIG-IC or MDA-5H. Taken together, these data suggested that the carboxyl-terminal amino acids 84 to 152 of US11 interacted with the carboxyl termini of RIG-I and MDA-5.

US11 interferes with the interaction between MAVS and RIG-I or MDA-5. The finding that the US11 protein binds RIG-I and MDA-5 and inhibits SeV-mediated activation of IFN-β suggests that US11 may block RIG-I and MDA-5 signaling to downstream adapters. MAVS acts downstream of RIG-I or MDA-5 in signal relay through complex formation with activated RIG-I or MDA-5. Co-IP analysis was performed to investigate whether US11 abrogates the interaction between MAVS and RIG-I or MDA-5. 293T cells were cotransfected with Myc-MAVS and either Flag-RIG-I or Flag-MDA-5 along with US11-HA or an empty vector. Whereas binding of MAVS to RIG-I or MDA-5 was readily demonstrated in the absence of the US11 protein (Fig. 6A and B), no interaction was seen in the presence of the US11 protein (Fig. 6A and B) or by nonspecific mouse monoclonal antibody IgG1 (Fig. 6A and B). To confirm that US11 interferes with the interaction between MAVS and RIG-I or MDA-5 during viral infection, 293T cells coexpressing Myc-MAVS and either Flag-RIG-I or Flag-MDA-5 were infected with WT or US11 mutant HSV-1 at an MOI of 10 and then subjected to co-IP analysis using anti-Myc MAb. In US11 mutant HSV-1-infected cells, RIG-I and MDA-5 were immunoprecipitated by MAVS using anti-Myc MAb (Fig. 6C and D) but not by nonspecific mouse monoclonal antibody IgG1 (Fig. 6C and D). In contrast, in WT HSV-1-infected cells, RIG-I and MDA-5 were not immunoprecipitated by MAVS using anti-Myc MAb (Fig. 6C and D). Collectively, these results demonstrate that US11 prevents the formation of the RIG-I/MAVS and MDA-5/MAVS complex in both transfected and HSV-1-infected cells.

US11 abrogates activation of IRF3 downstream of the RLR signaling pathway. RLR signaling activates transcription factors NF-κB and IRF3 via MAVS binding. The above data demonstrate that US11 can inhibit SeV-mediated activation of IFN-β and IRF3 promoter activities (Fig. 1). Subsequently, nuclear translocation of IRF3 was analyzed in HeLa cells transfected with or without an US11-HA expression plasmid by indirect immunofluorescence assay. In cells without SeV infection, IRF-3 localized exclusively to the cytoplasm and the expression of US11, which localizes in the cytoplasm and the nucleolus as demonstrated in our previous study (89), did not affect the expression or subcellular localization of IRF3 (Fig. 7A). In contrast, in SeV-infected cells, endogenous IRF3 translocated into the nucleus in more than 90% of cells (Fig. 7A and B), while ectopic expression of US11 abrogated the nuclear translocation of IRF3 induced by SeV infection (Fig. 7A); less than 10% of US11-expressing cells displayed nuclear accumulation of IRF3 (Fig. 7B). These results indicate that US11 can block the activation of IRF3 induced by SeV infection.

Generally, Ser 396 is targeted for phosphorylation following virus infection and plays an essential role in IRF-3 activation (76). Therefore, the phosphorylation state of IRF-3 following virus infection was evaluated by immunoblot analysis using the phospho-specific IRF-3 (S396) antibody. SeV infection induced the accumulation of Ser 396-phosphorylated IRF3 (Fig. 7C, lane 2), while US11 significantly abolished the Ser 396 phosphorylation induced by SeV infection. (Fig. 7C, lane 3). IRF-3 dimer formation is a consequence of IRF-3 phosphorylation. Thus, we tested whether the dimerization of IRF-3 induced by SeV infection could be inhibited by US11. IRF-3 dimerization was significantly reduced in SeV-infected cells expressing US11 (Fig. 7D). Taken together, these results demonstrate that US11 can impede IRF-3 activation by inhibiting its phosphorylation and dimerization.

DISCUSSION

Viral IFN antagonists are often multifunctional proteins, and their different properties may vary in importance at different stages of the virus replication cycle. HSV-1 US11 is an RNA-binding protein (6) that is expressed late in infection and packaged into the tegument layer of the virus particle as one of the most abundant viral proteins (31). As a tegument protein, US11 is delivered into the cytosol right after virus entry to perform a diverse array of tasks prior to the onset of viral gene expression (71). US11 has been reported to be a potent inhibitor of double-stranded-RNA-dependent PKR activation through binding to dsRNA (35) or through direct interaction with PKR in the context of viral infection (9) and therefore could interfere with the PKR-mediated host cell responses. PKR expression is stimulated by type I interferon during the cellular interferon-inducible antiviral response. Additionally, US11 has been recently shown to also counteract the activity of 2′-5′ OAS, a cellular protein critical for host cell defense (73). These observations suggest that US11 is a multifunctional protein and is involved in the viral immune evasion of host cell defense. Herein, in this study, we report that HSV-1 US11, as a novel viral IFN antagonist, binds to RIG-I and MDA-5 and inhibits its downstream activation of the RLR signaling pathway, preventing the transcriptional induction of IFN-β. These results provide further information on the mechanism by which HSV-1 US11 antagonizes the host antiviral response.

Small amounts of dsRNA are produced as a by-product of replication of most viruses, and thus, many viruses encode dsRNA-binding proteins that sequester dsRNA, thus preventing their detection by RIG-I and MDA-5, which is an important strategy for many viruses to evade the host IFN response. These proteins in-

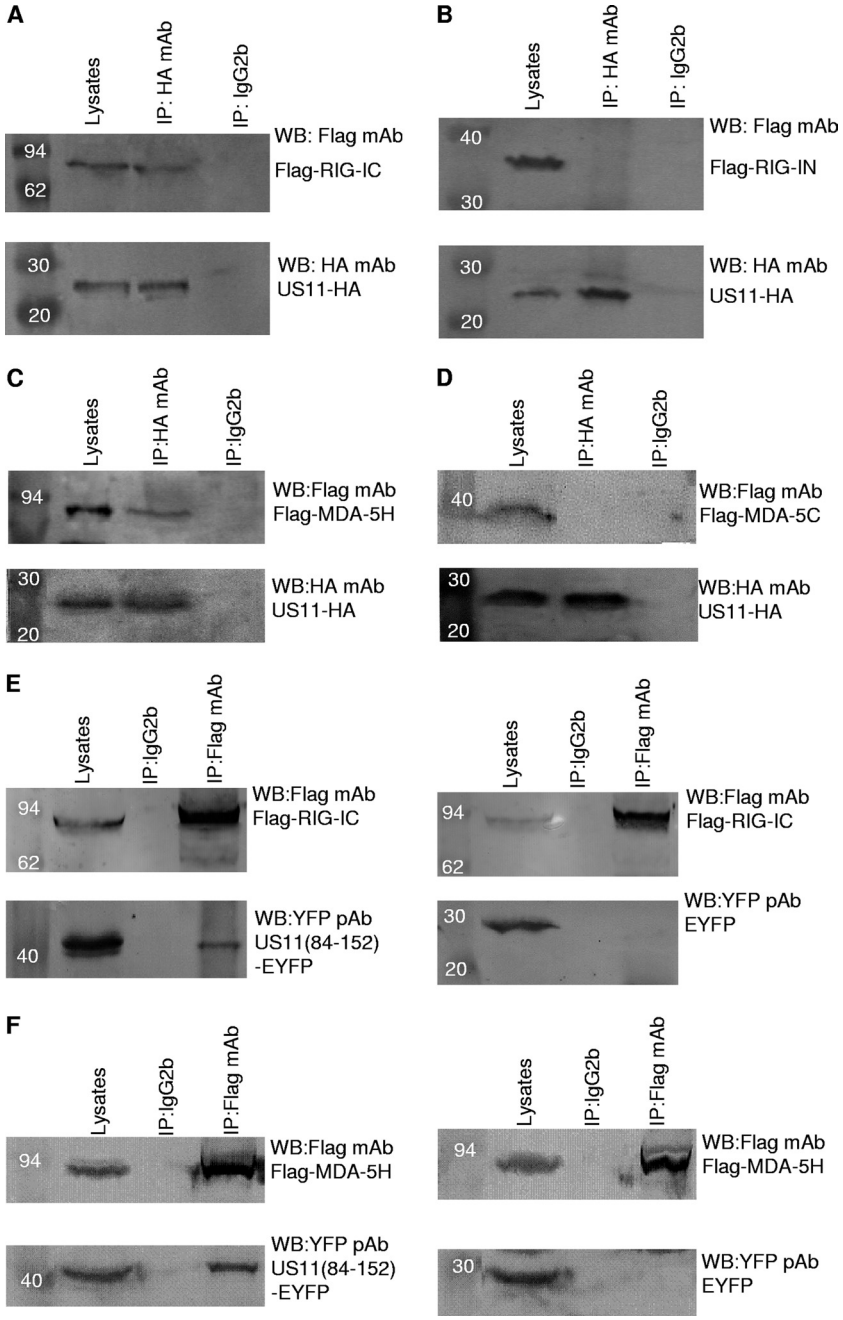

FIG 5 The carboxyl terminus of US11 interacts with the carboxyl termini of RIG-I and MDA-5. (A and B) HEK293T cells were cotransfected with plasmid pEF-Flag-RIG-IC (A) containing the carboxyl-terminal domain (aa 218 to 925) or pEF-Flag-RIG-IN (B) containing the amino-terminal domain (aa 1 to 229) and with plasmid US11-HA. (C and D) HEK293T cells were cotransfected with plasmid pEF-Flag-MDA-5H (C) containing the carboxyl-terminal helicase domain (aa 287 to 1025) or pEF-Flag-MDA-5C (D) containing the amino-terminal CARD domain (aa 1 to 287) and with plasmid US11-HA. Immunoprecipitation and Western blot analysis were performed as described for Fig. 5A. (E and F) HEK293T cells were cotransfected with plasmid pEF-Flag-RIG-IC (E) or pEF-Flag-MDA-5H (F) and with plasmid US11(84-152)-EYFP or pEYFP-N1, respectively. At 24 h after transfection, immunoprecipitation with anti-Flag MAb or nonspecific mouse monoclonal antibody (IgG2b) and Western blot analysis were performed as described for Fig. 5A.

clude vaccinia virus E3L (78, 87), Ebola virus VP35 (25), and influenza A virus NS1 (52) proteins. Interestingly, US11 has been shown to be a dsRNA-binding protein (35), which prompted us to investigate whether US11 has a similar ability to antagonize the host innate immune response. As anticipated, US11 could significantly inhibit the activation of the IFN-β promoter induced by SeV infection. Importantly, coinfection with US11 mutant HSV-1 showed less inhibition in SeV-induced mRNA accumulation and protein secretion of endogenous IFN-β than that of WT HSV-1. Furthermore, IFN-β secretion in WT HSV-1-infected cells without SeV infection was lower than that in US11 mutant HSV-1-infected cells, further demonstrating that US11 expression during HSV-1 infection contributes to inhibiting the production of IFN-β. In fact, we constructed another US11-Mut-BAC-Luc

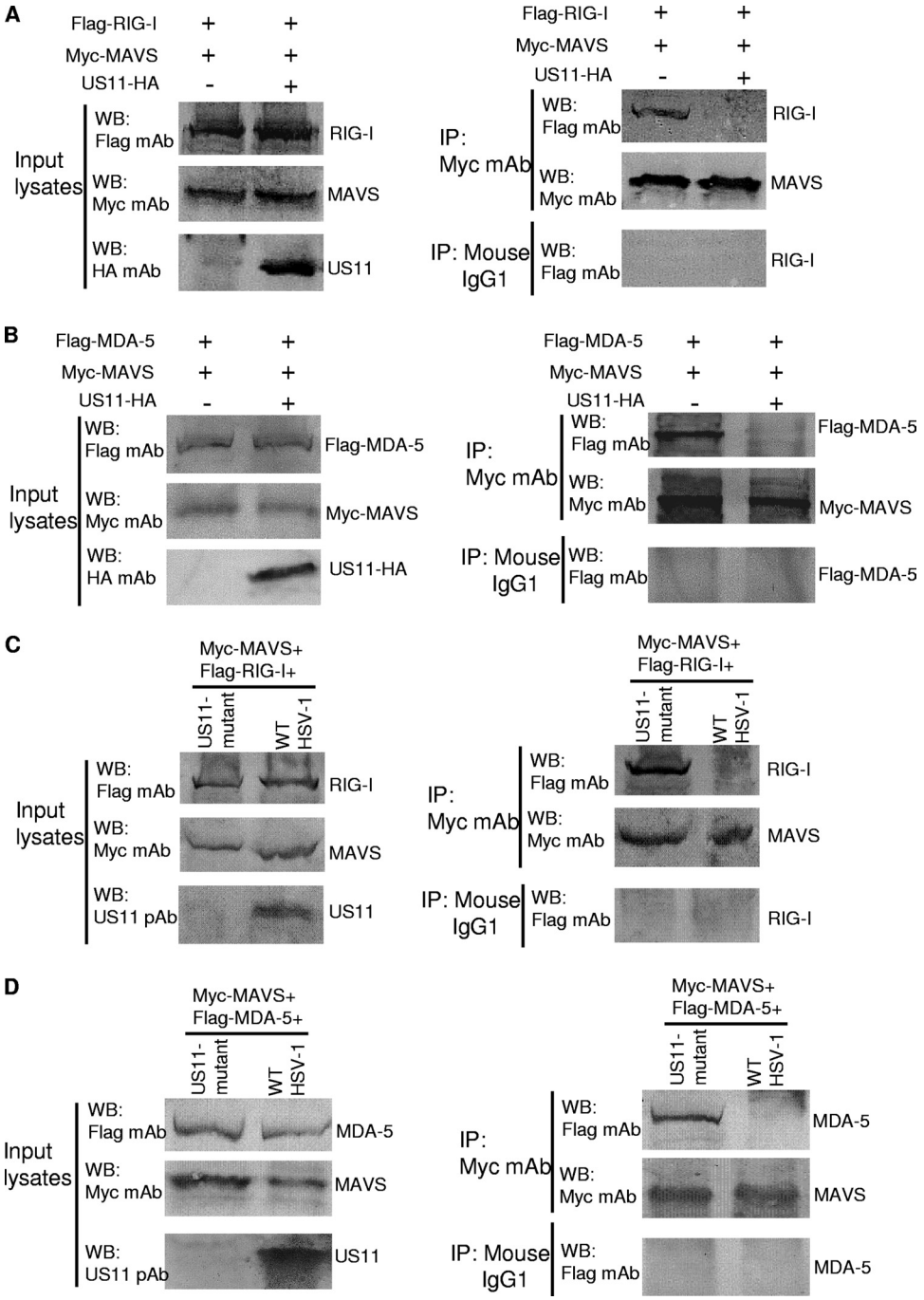

FIG 6 US11 blocks the formation of complex between RIG-I and MAVS or between MDA-5 and MAVS. (A and B) HEK293T cells ($\sim 5 \times 10^6$) were cotransfected with 10 μg of plasmid pEF-Flag-RIG-I (A) or pEF-Flag-MDA-5 (B) or 10 μg of plasmid pMyc-MAVS and 10 μg of plasmid US11-HA or transfected with empty vector. At 36 h posttransfection, cells were lysed and the clarified supernatants were subjected to immunoprecipitation assays using anti-Myc MAb (IP: Myc) or nonspecific mouse monoclonal antibody (IgG1). US11, MAVS, or RIG-I or MDA-5 was detected by Western blotting using anti-HA, anti-Myc, or anti-Flag MAbs, respectively. (C and D) HEK293T cells were cotransfected with plasmids pMyc-MAVS and pEF-Flag-RIG-I (C) or pEF-Flag-MDA-5 (D), respectively. Twenty hours after transfection, cells were infected with either WT or US11 mutant HSV-1 at an MOI of 10 for another 16 h. The cells were subsequently lysed and subjected to immunoprecipitation assays as described for panel A. Additionally, rabbit anti-US11 pAb was used for detection of US11 expression after either WT or US11 mutant HSV-1 infection.

HSV-1 virus (which is also lacking US11 expression) using our HSV-1 BAC system as previously described (44), and this virus showed properties identical to those of US11 mutant HSV-1 (data not shown). Interestingly, infection of HEK293T cells with a US11 mutant HSV-1 still downregulated the promoter activity of IFN-β induced by SeV, suggesting that other proteins of HSV-1 are also able to block activation of IFN-β. Indeed, it was previously reported that ICP0 (48, 51, 53, 60) and γ34.5 (56, 86) could antagonize type I IFN production, indicating that there are several proteins cooperatively responsible for immune evasion of host innate

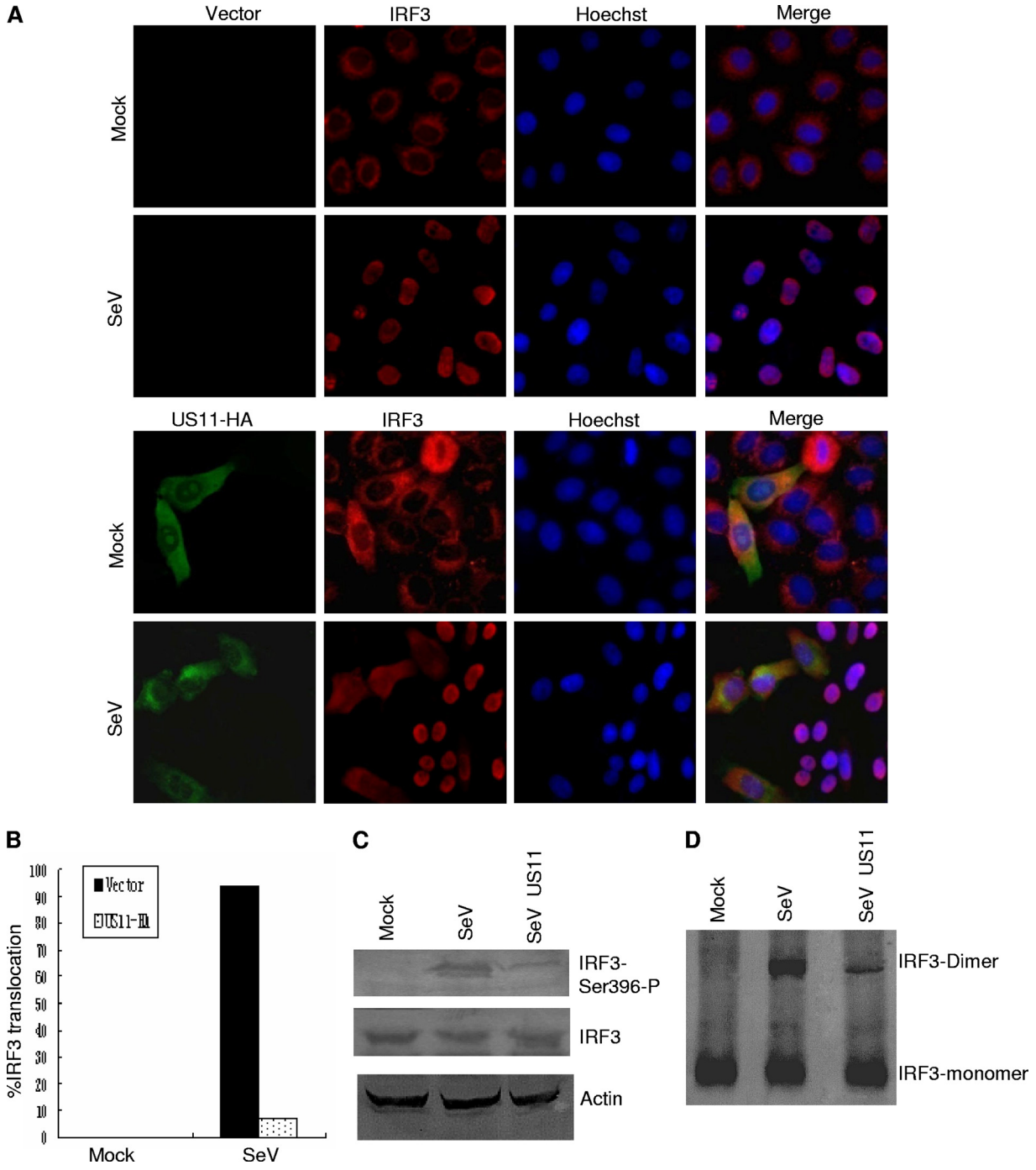

FIG 7 US11 impedes the activation of IRF3 downstream of the RLR signaling pathway. (A) US11 blocks IRF3 nuclear translocation induced by SeV infection. HeLa cells were transfected with the US11-HA expression plasmid or pCMV-HA vector. Twenty-four hours posttransfection, cells were then mock infected or infected with 100 HAU ml^{-1} SeV for 8 h. Cells were stained with mouse anti-HA MAb and rabbit anti-IRF3 antibody. FITC-conjugated goat anti-mouse (green) and TRITC-conjugated goat anti-rabbit (red) were used as the secondary antibody. Cell nuclei (blue) were stained with Hoechst 33258. The images were obtained by fluorescence microscopy using a ×40 objective. (B) Cells expressing US11-HA or vector from panel A were scored for nuclear translocation of IRF3. At least 100 cells were counted for each sample. Data shown are from one representative experiment of at least three. (C and D) US11 inhibits SeV-induced IRF3 phosphorylation and dimerization. HEK293T cells were transfected with the US11-HA expression plasmid. Twenty-four hours posttransfection, cells were then mock infected or infected with 100 HAU ml^{-1} SeV for 8 h. Protein extracts were subjected to SDS-PAGE (C) or native PAGE (D) for subsequent analysis with anti-phospho-IRF3 (Ser396) (C) or anti-IRF3 (D) antibody. IRF3 and actin as a loading control were also detected.

immunity in HSV-1. Moreover, ICP27 has been implicated in blocking subsequent IFN-mediated signaling (30). Subsequently, we found that US11 could not inhibit RIG-IN-, IPS-1-, TBK1-, IKKi-, or IRF-3/5D-mediated activation of IFN-β promoter activity (data not shown), suggesting that US11 might act at or upstream of the RIG-I level in the RIG-I signaling axis. Additionally, US11 could inhibit IFN-β promoter activity induced by overexpression of full-length MDA-5 (data not shown), indicating that

US11 may also block the IFN-β pathway at the MDA-5 level. We therefore hypothesized that US11 may interact with RIG-I or MDA-5 to inhibit IFN-β promoter activity.

To validate our hypothesis, co-IP analysis was employed to determine whether US11 could directly interact with RIG-I or MDA-5 under physiological conditions. As predicted, US11 interacted with both RIG-I and MDA-5 through its carboxyl-terminal domain harboring an RNA-binding domain in an RNA-independent fashion. Additionally, US11 was demonstrated to interact with RIG-I and MDA-5 in the context of an HSV-1 infection. In fact, the interaction of both US11 and RIG-I was also verified in a yeast two-hybrid system (data not shown). Additionally, we also demonstrated that US11 could prevent the formation of the RIG-I/MAVS and the MDA-5/MAVS complexes, which were confirmed following WT and US11 mutant HSV-1 infection. Therefore, we speculate that US11 binds to RIG-I and MDA-5 and then inhibits downstream activation of the RLR signaling pathway. Indeed, US11 not only could inhibit SeV-mediated activation of NF-κB promoter activity but also could impede IRF-3 activation by preventing its phosphorylation and dimerization. Thus, US11 binds to RIG-I and MDA-5 and abrogates their downstream signaling pathway of innate immunity.

Accumulating evidence demonstrates that RLRs are key components in mediating antiviral immunity in mammalian cells (33). It is therefore not surprising that viruses have evolved a variety of mechanisms to counteract RLR-mediated signaling of innate immunity. Many viruses, including RNA and DNA viruses, antagonize the host type I interferon response at the RIG-I level. For example, poliovirus, rhinoviruses, echovirus, and encephalomyocarditis virus 3C proteases cleave RIG-I (3, 4, 63), while the 3C protein of enterovirus 71 inhibits the RIG-I-mediated type I interferon response by interaction with RIG-I (40). Additionally, human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of IFN-β transcription via interaction with RIG-I (49). In the case of influenza A virus, NS1 is required for IFN-β antagonism, and this appears to be mediated by two independent mechanisms, one involving sequestration of dsRNA (20) and the second one mediated through an interaction with RIG-I (52). More recently, the Z proteins of New World arenaviruses were shown to bind RIG-I and interfere with type I interferon induction (23). Furthermore, several paramyxoviruses encode a V protein that interacts with MDA-5 (2). Interestingly, some viruses encode proteins that bind dsRNA and prevent their detection by RIG-I and MDA-5 in the cytoplasm. These include the vaccinia virus E3L protein (78, 87), the Ebola virus VP35 protein (25), and the human cytomegalovirus proteins TRS1 and m142/m143 (8, 13, 14). In this study, we identified US11 as a novel antagonist of virus-mediated signaling, which could also bind to RIG-I and MDA-5 and inhibit downstream activation of the RLR signaling pathway. It was previously reported that US11 could bind to RNA, including dsRNA (6, 35), and thus, US11 binding to RNA might potentially sequester RNA from being recognized by both RIG-I and MDA-5. However, the most likely scenario is that both actions inherent to the carboxyl domain of the US11 protein are involved in mediating antiviral activity; this possibility remains to be tested in the future.

The precise mechanism by which HSV-1 US11 inhibits the activity of RIG-I and MDA-5 remains to be determined. However, several recent studies provide insights into the potential mechanism. It is reported that Riplet/RNF135 binds and ubiquitinates the C-terminal region of RIG-I to promote RIG-I-mediated IFN-β promoter activation (59). More recently, studies have shown that the dsRNA-binding protein PACT could bind to the C-terminal repression domain of RIG-I to facilitate innate antiviral responses (38). Interaction of the US11 protein with RIG-I may disrupt RIG-I interaction with any of the aforementioned proteins. Future work will focus on fine mapping the interaction between RIG-I and US11 in an effort to elucidate the precise mechanism of US11 for antagonizing the host innate antiviral response. It was reported that all paramyxovirus V proteins inhibit activation of MDA-5 by blocking dsRNA binding and consequent self-association. Thus, we speculate that US11 may inhibit activation of MDA-5 by blocking its self-association.

Although the RLRs are sensors of RNA, some data have suggested a role for this system in the detection of HSV-1 DNA (12). AT-rich DNA can be transcribed by RNA polymerase III into 5′-triphosphate RNA, which subsequently activates RIG-I. This indirect DNA-sensing system was reported to be involved in the induction of type I IFNs following HSV-1 infection (16). However, other studies have not been able to show a role for RNA polymerase III in sensing HSV-1 DNA (50, 84), and one report has shown direct interaction between HSV-1 DNA and RIG-I and nonredundant roles for RIG-I and MDA-5 in sensing HSV DNA in fibroblasts (17). These findings raise questions as to the requirement for RNA polymerase III in herpesvirus DNA recognition and urge identification of the potential mechanism involved. Additionally, it was reported recently that HSV-1-induced IFN responses by primary human monocyte-derived macrophages depend on MDA-5, but not RIG-I, and its adapter protein MAVS (50). Interestingly, we found that US11 could interact with MDA-5 and inhibit MDA-5-mediated IFN-β production, which may help HSV-1 to evade the early host antiviral response.

US11, as an antagonist of IFN-β production, may be one of several strategies used by HSV-1 to interrupt the innate immune system. However, more work should be done to reveal the comprehensive tricks by HSV-1 to subvert the host immune response. In conclusion, we have demonstrated that the US11 protein binds to RIG-I and MDA-5 and inhibits downstream activation of the RLR signaling pathway. To our knowledge, this is the first time that HSV-1 has been demonstrated to interfere with RIG-I and MDA-5, and thus these findings reveal a novel mechanism for HSV-1 to evade the host antiviral response.

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