Modulation of Mitochondrial Antiviral Signaling by Human Herpesvirus 8 Interferon Regulatory Factor 1

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ABSTRACT

Mitochondrial lipid raft-like microdomains, experimentally also termed mitochondrial detergent-resistant membrane fractions (mDRM), play a role as platforms for recruiting signaling molecules involved in antiviral responses such as apoptosis and innate immunity. Viruses can modulate mitochondrial functions for their own survival and replication. However, viral regulation of the antiviral responses via mDRM remains incompletely understood. Here, we report that human herpesvirus 8 (HHV-8) gene product viral interferon regulatory factor 1 (vIRF-1) is targeted to mDRM during virus replication and negatively regulates the mitochondrial antiviral signaling protein (MAVS)-mediated antiviral responses. The N-terminal region of vIRF-1 interacts directly with membrane lipids, including cardiolipin. In addition, a GxRP motif within the N terminus of vIRF-1, conserved in the mDRM-targeting region of mitochondrial proteins, including PTEN-induced putative kinase 1 (PINK1) and MAVS, was found to be important for vIRF-1 association with mitochondria. Furthermore, MAVS, which has the potential to promote vIRF-1 targeting to mDRM possibly by inducing cardiolipin exposure on the outer membrane of mitochondria, interacts with vIRF-1, which, in turn, inhibits MAVS-mediated antiviral signaling. Consistent with these results, vIRF-1 targeting to mDRM contributes to promotion of HHV-8 productive replication and inhibition of associated apoptosis. Combined, our results suggest novel molecular mechanisms for negative-feedback regulation of MAVS by vIRF-1 during virus replication.

IMPORTANCE

Successful virus replication is in large part achieved by the ability of viruses to counteract apoptosis and innate immune responses elicited by infection of host cells. Recently, mitochondria have emerged to play a central role in antiviral signaling. In particular, mitochondrial lipid raft-like microdomains appear to function as platforms in cell apoptosis signaling. However, viral regulation of antiviral signaling through the mitochondrial microdomains remains incompletely understood. The present study demonstrates that HHV-8-encoded vIRF-1 targets to the mitochondrial detergent-resistant microdomains via direct interaction with cardiolipin and inhibits MAVS protein-mediated apoptosis and type I interferon gene expression in a negative-feedback manner, thus promoting HHV-8 productive replication. These results suggest that vIRF-1 is the first example of a viral protein to inhibit mitochondrial antiviral signaling through lipid raft-like microdomains.

Human herpesvirus 8 (HHV-8), also called Kaposi's sarcoma-associated herpesvirus (KSHV), is a pathogenic DNA virus associated with Kaposi's sarcoma (KS) and the B cell malignancies primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), which often occur in immunocompromised individuals, such as those with human immunodeficiency virus type 1 (HIV-1) infection or undergoing organ transplantation (1, 2). Virus productive replication, in addition to latency, is important for maintaining viral load within the host and also for HHV-8-associated pathogenesis. Successful virus replication is in large part achieved by the ability of viruses to counteract antiviral responses of the host cells, such as apoptosis and innate immune responses. HHV-8 encodes a number of proteins expressed during the lytic cycle that have demonstrated or potential abilities to promote virus productive replication via inhibition of apoptosis and innate immune signaling pathways (3). Among them, viral interferon (IFN) regulatory factor 1 (vIRF-1) is believed to play crucial roles in blocking interferon and other stress responses to virus infection and replication by negatively interacting with cellular stress signaling proteins, including p53, ATM, IRF-1, IRF-3, GRIM19, SMAD3, and SMAD4 (3–5). In addition, we discovered that vIRF-1 localizes to the outer mitochondrial membrane (OMM) and inhibits the mitochondrial intrinsic apoptosis pathway via its inhibitory interaction with proapoptotic BH3-only

proteins (BOPs), including Bim and Bid. This inhibitory interaction is important for promoting viral productive replication (6, 7). However, the molecular mechanism of mitochondrial localization of vIRF-1 and the precise role of mitochondria-targeted vIRF-1 are not well understood.

The primary function of mitochondria is to produce energy in the form of ATP through the process of oxidative phosphorylation. In addition, mitochondria play crucial roles in fatty acid metabolism, lipid trafficking, and calcium buffering (8). Furthermore, recent studies have demonstrated that mitochondria play a central role in the antiviral signaling pathways leading to apoptosis and innate immunity (9–12). For example, proapoptotic proteins, such as BOPs, are elevated and/or activated during virus

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replication. BOPs induce mitochondrial outer membrane permeabilization, a crucial step in the intrinsic apoptotic process that triggers the release from the intermembrane space of soluble apoptotic factors, such as cytochrome *c* (6). In response to viral infection, the RIG-I-like receptors (RLRs) RIG-I and MDA-5 recognize cytosolic viral RNA and activate the mitochondrial antiviral signaling protein (MAVS; also known as IPS-1, VISA, and Cardif), which recruits TBK1 and I κ B kinase γ (IKK γ) kinases to activate IRF-3 and IRF-7 transcription factors. IRF-3 and IRF-7 activation leads to the expression of type I IFN genes that restrict virus replication (11, 12). Thus, successful virus infection and replication are linked to the ability of the virus to inhibit antiviral responses mediated by mitochondria. For example, human herpesviruses encode antiapoptotic proteins to inhibit the intrinsic apoptosis pathway (10), and hepatitis C virus encodes a serine protease, NS3/NS4A, to disrupt RLR signaling and IFN- β production by cleaving MAVS from the OMM (13). Furthermore, the severe acute respiratory syndrome coronavirus encodes a non-structural protein, NSP15, which inhibits MAVS-induced apoptosis (14). Interestingly, MAVS was reported to inhibit the production of infectious virions in HHV-8-infected cells (15), although whether and how HHV-8 might counteract MAVS-mediated antiviral signaling remained unclear.

Here, we show that vIRF-1 is recruited to mitochondrial lipid raft-like microdomains, termed mitochondrial detergent-resistant microdomains (mDRM), by directly interacting with membrane lipids, including cardiolipin (CL), via the vIRF-1 N-terminal region. vIRF-1 also interacts with MAVS and inhibits MAVS aggregation, which is essential for its antiviral activity (i.e., both type I IFN induction and apoptosis). Furthermore, we demonstrated that vIRF-1 targeting to mDRM is dependent on MAVS-induced generation of mitochondrial reactive oxygen species (mROS), correlates with cardiolipin externalization, and is essential for apoptosis inhibition and successful HHV-8 productive replication. Thus, HHV-8 vIRF-1 appears to represent the first example of a viral protein targeting to the mDRM and negatively regulating MAVS-mediated antiviral responses.

MATERIALS AND METHODS

Plasmids and oligonucleotides. Plasmid expressing vIRF-1 was described previously (7). The cDNA of vIRF-1 Δ PD lacking residues 1 to 75 was PCR amplified and inserted between the BamHI and EcoRI sites of pcDNA3.1/Flag (6). Subsequently, the cDNAs of PTEN-induced putative kinase 1 (PINK1) and TOM20 mitochondrial targeting sequences (MTSSs) were PCR amplified and inserted between HindIII and BamHI of the pcDNA3.1/vIRF-1 Δ PD-Flag plasmid. The open reading frame of vBcl-2 was obtained from lytic BCBL-1 cells and cloned into pcDNA3.1/Flag. vIRF-1 and MAVS short hairpin RNAs (shRNAs) were cloned into lentiviral pYNC352/puro or GFP-puro vector using BamHI and MluI sites as described previously (6); sequences are as follows: for vIRF-1 shRNA, 5'-GGCATTCTGCTGACTAGCTCT-3'; for MAVS shRNA1, 5'-TAAGTATATCTGCCGCAATTT-3'; and for MAVS shRNA2, 5'-CTGCCGCAATTT-3'. For lentiviral transduction of vIRF-1 and its variants along with either control NS or vIRF-1 shRNA, the corresponding cDNAs were cloned into the transcription unit under the control of the polyubiquitin *c* (UBC) promoter in the pDUET.Hyg vector using BamHI and XhoI enzyme sites. The units were transferred to the lentiviral pYNC352 shRNA vectors using PacI and XhoI enzyme sites. The oligonucleotide sequences used to generate the vIRF-1^R variant resistant to the vIRF-1 shRNA is 5'-GGAATCCTACTACGAGTTCC-3' (mutated bases are underlined). For CRISPR/Cas9-mediated targeted genome mutagenesis, a guide RNA (gRNA) specific to the MAVS gene was designed using

the E-CRISP tool (16) and cloned into the LentiCRISPR-V2 vector using the BsmBI enzyme site as previously described (17, 18). The MAVS gRNA sequences are 5'-CAGGGAACCGGGACACCCUC-3'. For the expression of GFP-fused vIRF-1 proteins in mammalian cells, vIRF-1 DNA fragments were PCR amplified and subcloned between the EcoRI and BamHI sites of pEGFP-N1 (Clontech). For the expression of green fluorescent protein (GFP)-fused proteins in bacteria, DNA fragments encoding the recombinant proteins were inserted between the NheI and SalI sites of pTYB4-EGFP (6). PINK1-Myc (plasmid 13314) and LentiCRISPR-V2 (plasmid 52961) were purchased from Addgene. Flag-MAVS, Δ RIG-I-GFP, and IFN- β -luc reporter plasmids were obtained from Edward Harhaj. All PCR amplification and site-directed mutagenesis were performed using Platinum Pfx DNA polymerase. The sequences of primers used for HHV-8 genome copy number analysis were as follows: for LANA, 5'-TACGGTTGGCGAAGTCACATC-3' (forward) and 5'-CCTCGCAGCA GACTACACCTCCAC-3' (reverse), and for GFP, 5'-AAGCTGACCTCG AAGTTCATCTGC-3' (forward) and 5'-CTTGTAAGTTGCCGTCGCTCTTGAA-3' (reverse).

Antibodies and reagents. Antibodies against calregulin (H-170), ACSL4 (F-4), prohibitin (H-80), Bax (N-20), VDAC1 (20B12), MAVS (E-3), LDH (H-160), GFP (FL), caspase-8 (8CSP03), heat shock protein 60 (HSP60) (B-9), FLOT1 (C-2), TOM20 (F-10), and Jun N-terminal protein kinase 1 (JNK1) (F-3) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase-8 (18C8), poly-(ADP-ribose) polymerase (PARP) (46D11), phospho-JNK1/2 (Thr183/Tyr185), and catalase (D4P7B) were purchased from Cell Signaling Technology (Danvers, MA). Anti-Myc (OP-10) was purchased from EMD Millipore (Billerica, MA). Rabbit anti-MAVS polyclonal antibody (14341-1-AP) was purchased from Proteintech (Chicago, IL). Anti-cytochrome *c* (EP1327) antibody was purchased from Abcam (Cambridge, MA). Anti-Flag (M2) and anti- β -actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Human anticardiolipin antibody (C1375) was purchased from United States Biological (Salem, MA). Rabbit anti-vIRF-1 antiserum was a gift of Gary Hayward. MitoTEMPO (SML0737) was purchased from Sigma. MitoQ (BN0860) was purchased from Biotrend Chemicals (Destin, FL).

Cell culture, transfections, and virus infections. BCBL-1 TRE:RTA cells were maintained in RPMI 1640 supplemented with 10% tetracycline-free fetal bovine serum (FBS) and treated with 1 μ g/ml of doxycycline (DOX) to induce the reactivation of HHV-8. HeLa, 293T, and 293 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS and antibiotics. Plasmid DNA transfection was performed using GenJet version II (SigmaGen Laboratories, Rockville, MD). Lentiviral transduction into BCBL-1 cells was performed by spinoculation at 800 $\times$ g for 30 min with multiplicities of infection (MOIs) of 5 to 10. For the infection of vesicular stomatitis virus (VSV) (19, 20), 293T or HeLa cells were serum starved for 1 h, inoculated with VSV in serum-free DMEM at the desired MOI for 1 h, and further incubated in complete DMEM for the times indicated below. For generation of MAVS knockout (KO) cells, 293T cells were stably transfected with LentiCRISPR-V2-MAVS gRNA in the presence of puromycin and subjected to cell cloning by limiting dilution.

Immunological analyses. For immunoblotting, total cell or mitochondrial extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with appropriate antibodies diluted in SuperBlock (phosphate-buffered saline [PBS]) blocking buffer (Thermo Scientific, Rockford, IL). The immunoreactive bands were visualized by enhanced chemiluminescence (ECL) reagents (Perkin-Elmer). For the immunoprecipitation with DRM, cells were lysed in TNE buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 1 mM EDTA) supplemented with 1% Triton X-100 and protease inhibitor cocktail on ice and centrifuged at 20,000 $\times$ g for 10 min. The pellet was resuspended in RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Igepal CA-630, and 0.25% deoxycholate) and sonicated for 10 min in ice water at a high power setting (320 W) using Bioruptor (Diagenode, Denville, NJ). Sonicated

samples were cleared of debris by centrifugation, incubated at 4°C overnight with the desired antibodies, and incubated with protein A-agarose beads for an additional 3 h. Immunoprecipitates were washed with RIPA buffer, followed by elution of bound proteins with 1.5× SDS sample buffer or 3× Flag peptide (Sigma, St. Louis, MO). For immunostaining, cells were fixed and permeabilized in chilled methanol for 10 min. BCBL-1 cells were immobilized on a poly-L-lysine-coated coverslip. For detection of mitochondrial superoxide, cells were incubated with 5 μM MitoSOX red indicator (Invitrogen) in Hanks' buffered salt solution (HBSS) containing calcium and magnesium for 10 min at 37°C just before cell fixation. Stained cells were imaged on Nikon E-800 with a 60× oil-corrected objective and Element software.

Subcellular fractionation. Pure mitochondria were isolated using Axis-Shield OptiPrep iodixanol (Sigma-Aldrich, St. Louis, MO) as previously described (21). Briefly, cultured cells were homogenized in buffer B (0.25 M sucrose, 1 mM EDTA, 20 mM HEPES-NaOH [pH 7.4]) with 30 strokes of a Dounce glass homogenizer and centrifuged at $1,000 \times g$ for 10 min. An aliquot of homogenate was used as total cell extracts. The supernatant was further centrifuged at $13,000 \times g$ for 10 min. The pellet was used as a crude fraction enriched in mitochondria. For further enrichment, the pellet was resuspended in 36% iodixanol, bottom-loaded under 10% and 30% iodixanol gradients, and centrifuged at $50,000 \times g$ for 4 h. The mitochondria were collected at the 10%/30% iodixanol interface. Alternatively, to separate mitochondrion-associated endoplasmic reticulum (ER) membranes (MAM) from the crude mitochondrion-enriched fraction, a self-generating Percoll gradient (30%) was employed as described previously (22). For isolation of mDRM, crude or pure mitochondria were incubated in the TNE buffer containing 1% Triton X-100 (or other detergents as indicated) on ice for 30 min, except for 2% SDS at room temperature, and centrifuged at $21,000 \times g$ for 10 min. The supernatant was used as a soluble mitochondrial fraction, and the pellet was used as the mDRM fraction. The pellet was boiled in 1× SDS sample buffer. For further enrichment, the pellet was resuspended in 40% iodixanol, bottom-loaded under 5% to 30% iodixanol gradients, and centrifuged on a 5%/30% iodixanol gradient at $260,000 \times g$ for 20 h at 4°C. Each fraction was collected from the top of tube and analyzed by immunoblotting.

Purification of recombinant proteins. Recombinant GFP or GFP-fused proteins fused to intein-chitin binding domain in pTYB4 were expressed in Rosetta (DE3) cells (Novagen) and purified as previously described (6). Purified proteins were visualized using a colloidal blue staining kit (Invitrogen).

In vitro mitochondrial binding assay. Isolated 293T mitochondria were blocked with buffer B containing 5% fatty acid-free bovine serum albumin (BSA) for 1 h at 4°C and then incubated with recombinant GFP or GFP fusion proteins in the blocking buffer for 1 h. After being washed with buffer B three times, mitochondrial pellets were boiled in 1× SDS sample buffer and analyzed by immunoblotting.

Lipid binding assays. Protein lipid overlay assay was performed as described by the manufacturer (Echelon Biosciences, Salt Lake City, UT). Briefly, membrane lipid strips (P-6002) were blocked with PBS buffer containing 0.1% Tween 20 and 3% fatty acid-free BSA and then incubated with 1 μg/ml of GFP or GFP-fused vIRF-1 (residues 1 to 153) proteins for 1 h at room temperature. After a washing, the membranes were incubated with rabbit anti-GFP antibody and subsequently secondary anti-rabbit IgG-horseradish peroxidase (HRP) antibody. Immunoreactive lipid dots were visualized with ECL reagents. For enzyme-linked immunosorbent assay (ELISA)-based cardiolipin binding assay, a Corning 96-well enzyme immunoassay/radioimmunoassay (EIA/RIA) plate was coated with cardiolipin (C1649; Sigma) in the dark at 4°C overnight and blocked in assay buffer (10 mM bis-Tris and 10 mM CaCl₂) containing with 0.5% skim milk and 2% fatty acid-free BSA for 1 h as previously described (23). GFP or GFP-fused vIRF-1 proteins were applied to wells in triplicate and incubated for 1 h. After a washing, anti-GFP antibody was added to wells and incubated for 30 min. Detection was performed with secondary anti-

rabbit IgG-HRP and one-step Ultra TMB-ELISA substrate solution (Thermo Scientific).

Quantitation of externalized cardiolipin. Crude mitochondria isolated from cells transfected or stimulated were incubated with blocking buffer (homogenization buffer B containing 5% fatty acid-free BSA and normal rabbit IgG [5 μg/ml]) at 4°C for 1 h. Then, human anticardiolipin antibody was added to the mitochondrial suspension and incubated on ice for 30 min with gentle rocking. After gentle washing with buffer B three times, the mitochondrial pellet was boiled in 1× SDS sample buffer and analyzed by immunoblotting using goat anti-human IgG-HRP (A18811; Invitrogen).

SDD-AGE. Semidenaturing detergent agarose gel electrophoresis (SDD-AGE) was performed according to a published protocol, with minor modifications (24). Briefly, crude mitochondria were resuspended in 1× sample buffer (0.5× Tris-borate-EDTA [TBE], 10% glycerol, 2% SDS, and 0.0025% bromophenol blue) and loaded onto a 1.5% agarose gel. After electrophoresis in the running buffer (1× TBE and 0.1% SDS) for 1 h with a constant voltage of 100 V at 4°C, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane for immunoblotting.

Cell viability assay. The CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI), which quantifies ATP as a measure of metabolically active cells, was used to measure cell viability.

Luciferase reporter assay. Cells were transfected with the desired plasmids together with IFN-β-Luc and the *Renilla* reporter pTK-RLuc as an internal control. After 24 h, cells were lysed in passive lysis buffer (Promega) and subjected to dual-luciferase assay as recommended by the manufacturer (Promega). Results are presented as the relative firefly luciferase activity over the *Renilla* luciferase activity.

HHV-8 replication assay. For determination of encapsidated HHV-8 genome copy number, viral DNA was purified using PureLink viral RNA/DNA minikit (Invitrogen) following pretreatment of virus suspension with DNase I at 37°C overnight. An aliquot of VSV-GFP was added as an internal control to the HHV-8 suspension and subjected to reverse transcriptase reaction. Quantitative PCR (qPCR) was performed in a 96-well microplate using an ABI Prism 7500 detection system (Applied Biosystems, Foster City, CA) with RT²Real-Time SYBR green/ROX master mix (Qiagen, Valencia, CA). Reactions were performed in a total volume of 25 μl, containing viral DNA sample and a 250 nM concentration of a pair of primers specific for LANA or GFP genes. To calculate the copy number of viral DNA, a plasmid encoding LANA or GFP was used as a standard.

Statistical analyses. Statistical data analysis was performed using Kaleidagraph Synergy software (version 4.5). Error bars in figures represent standard deviations (SD). Significance was determined by two-tailed, unpaired Student's *t* test with a confidence level of 95%.

RESULTS

vIRF-1 localizes to the mitochondria by targeting to mDRM. Previously, we found that vIRF-1 localized to the mitochondria in HHV-8-infected cells and inhibited BOP-induced apoptosis (6). vIRF-1 lacks any typical mitochondrial targeting sequences (MTSs). Also, the mitochondrial localization of vIRF-1 was not dependent on its interaction with BOPs (6), implying that vIRF-1's mitochondrial localization may be mediated by either an atypical MTS or a direct interaction with other mitochondrial molecules. In an attempt to identify vIRF-1-interacting mitochondrial proteins, we initially conducted immunoprecipitation assays but were unable to detect vIRF-1 in an *n*-dodecyl β-D-maltoside (DDM)-soluble mitochondrial fraction derived from lytically reactivated PEL cells, BCBL-1 cells, infected with HHV-8 (Fig. 1A). Similarly, mitochondrial vIRF-1 was not readily solubilized in other non-ionic detergents, including digitonin, NP-40, Triton X-100, and the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), while other mitochondrial proteins, prohibitin and Bax, were detectable in the soluble

FIG 1 vIRF-1 targets to mDRM. (A) Detergent solubility of vIRF-1 in mitochondria. The mitochondria isolated from reactivated BCBL-1 cells were incubated in TNE buffer containing the detergents *n*-dodecyl β -D-maltoside (DDM), digitonin, NP-40, Triton X-100, CHAPS, and SDS, and the supernatant (Sup) and pellet (Pel) fractions separated by centrifugation were immunoblotted with the indicated primary antibodies. (B) Identification of vIRF-1 in mDRM. The Triton X-100-insoluble fraction of the mitochondria derived from reactivated BCBL-1 cells was further fractionated on iodixanol gradients, 5% to 40%, and each fraction was collected and immunoblotted with antibodies to vIRF-1 and mDRM-localized voltage-dependent anion channel 1 (VDAC1).

fractions (Fig. 1A). Instead, mitochondrial vIRF-1 was detected in the detergent-insoluble pellet fractions (Fig. 1A). Surprisingly, vIRF-1 was only partially solubilized, even with 1% SDS at room temperature, while prohibitin and Bax were fully solubilized (Fig. 1A), indicating that mitochondrial vIRF-1 is highly resistant to detergents. Recent experimental evidence showed that mitochondria contain lipid raft-like microdomains (mDRM) (25). Accordingly, we further fractionated the Triton X-100-insoluble mitochondrial fraction on a 5 to 40% iodixanol gradient to determine if vIRF-1 was localized to these microdomains. Indeed, vIRF-1 was detected in one major peak, which comigrated with voltage-dependent anion channel 1 (VDAC1), a marker of mDRM (Fig. 1B). Here, the term mDRM is used to refer to the Triton X-100-insoluble pellet fraction. Taken together, these results suggest that vIRF-1 localizes to mitochondria by targeting to mDRM.

The PD of vIRF-1 is required and sufficient for mDRM targeting. To identify regions of vIRF-1 required for mDRM targeting, we used a series of deletion mutants in which blocks of 25 residues were deleted from vIRF-1 and examined their detergent solubilities in mitochondria (Fig. 2A). Deletion of each of the first three blocks, encompassing the proline-rich domain (PD), significantly reduced detection of vIRF-1 in mDRM, while the protein levels of flotillin-1 (FLOT1), which was used as a loading control and a mDRM marker (26, 27), remained constant. These results suggest that mDRM-targeting sequences are contained within these segments or that they are required for the formation of a domain conformation appropriate for mDRM targeting. Next, to determine if the vIRF-1 PD was sufficient for mDRM targeting, we performed an mDRM targeting assay using the mitochondria isolated from 293T cells transfected with GFP or GFP-fused vIRF-1 PD encompassing residues 1 to 75 (PD-GFP). Indeed, the PD increased the detergent insolubility of GFP in mitochondria, while the levels of FLOT1 remained constant in mDRM (Fig. 2B). This result suggests that the vIRF-1 PD is likely to be anchored in the mitochondrial membrane. To test this, we washed the mitochondria isolated from 293T cells expressing vIRF-1 PD-GFP or GFP with a high concentration of sodium carbonate (0.1 M; pH 11.5), which strips off peripheral proteins from the membranes. Indeed, PD-GFP was detected only in the pellet fractions after a washing with alkaline sodium carbonate, whereas GFP was barely detected in either the mitochondrial soluble or pellet fraction (Fig. 2C), indicating that vIRF-1 PD is integrated into the mitochondrial

membranes. Taken together, these results suggest that the mitochondrial localization of vIRF-1 involves integration of the PD region into mDRM.

The vIRF-1 PD has sequence homology with PINK1 MTS. Next, we compared the primary protein structure of vIRF-1 PD with that of other mDRM-targeted proteins using the Basic Local Alignment Search Tool (BLAST) and identified two regions in vIRF-1 PD that were homologous to a segment of the MTS of PTEN-induced putative kinase 1 (PINK1) (Fig. 3A). PINK1, also known as PARK6, is a mitochondrial serine/threonine kinase involved in mitochondrial quality control. Thus far, the mDRM targeting region of PINK1 has not been defined precisely. To determine if the vIRF-1 homology domain of PINK1 is involved in mDRM targeting, we generated a PINK1 deletion mutant (Δ 32-60) lacking residues 32 to 60 and performed an mDRM targeting assay. The mutated protein was abrogated in mDRM targeting relative to wild-type (wt) PINK1 (Fig. 3B). To test whether the PINK1-MTS is functionally equivalent to the vIRF-1 PD with respect to mDRM targeting, the MTS of PINK1 was fused to PD deletion vIRF-1 (Δ PD, amino acids [aa] 76 to 449) (Fig. 3C). While vIRF-1 Δ PD was barely detected in mDRM, PINK1³²⁻⁷⁷- Δ PD and also PINK1¹⁻⁷⁷- Δ PD and PINK1¹⁻³⁶- Δ PD were detected in mDRM at high levels, and the cleavage products of the last two were also detected (Fig. 3C). This cleavage is consistent with previous reports that PINK1 is cleaved at two potential residues, residues 35 and 77, presumably by a mitochondrial processing peptidase (28). Taken together, these results suggest that the sequences for mDRM targeting are conserved between vIRF-1 and PINK1. In addition, we noted that a GxRP motif was present in vIRF-1 (GQRP, residues 4 to 7), MAVS (GPRP, residues 492 to 495) and PINK1 (GERP, residues 43 to 46) (Fig. 3D); for MAVS, this is located within the region shown to be necessary for targeting of MAVS to mDRM (29). An *in vitro* mitochondrial binding assay showed that a PD-GFP mutant in which the GQRP motif was mutated to AQAA could not localize to the mitochondria (Fig. 3E). These data identify a novel mitochondrial localization motif within the PD of vIRF-1.

vIRF-1 binds to membrane lipids, including cardiolipin. To further understand how the vIRF-1 PD associates with mDRM, we tested if vIRF-1 PD binds directly to membrane lipids using the protein lipid overlay (PLO) assay, in which membrane lipid strips were probed with purified recombinant control GFP and GFP-

FIG 2 The proline-rich domain (PD) is required and sufficient for vIRF-1 targeting to mDRM. (A) Mapping of the mDRM targeting regions of vIRF-1. Each of 18 blocks of 25 amino acids (aa) was deleted from the N-terminal end of vIRF-1. The mDRM fractions and total cell extracts derived from 293T cells transfected with Flag-tagged full-length vIRF-1 (FL) or 18 deletion mutants were immunoblotted (IB) with the indicated antibodies. Flotillin-1 (FLOT1) and lactate dehydrogenase (LDH) were used as loading controls for mDRM and total cell extracts, respectively. (B) mDRM targeting of vIRF-1 PD alone. Total cell extracts (T) and mitochondrial detergent-soluble (S) and -insoluble (P) fractions derived from 293T cells transfected with GFP or GFP-fused vIRF-1 PD (PD-GFP) were immunoblotted with primary antibodies to GFP or FLOT1. (C) Membrane integration of vIRF-1 PD. The mitochondria isolated from 293T cells transfected with GFP or PD-GFP were washed in 0.1 M sodium carbonate (Na_2CO_3 , pH 11.5) for 30 min and then centrifuged at $17,000 \times g$ for 10 min. The supernatant (S) and pellet (P) fractions were immunoblotted with anti-GFP antibody. The expression levels of GFP and PD-GFP proteins were examined by anti-GFP immunoblotting of total cell extracts.

fused vIRF-1 PD proteins (Fig. 4A). Initially, we were unable to reliably identify a vIRF-1 PD-binding lipid due to low signals (data not shown). As an alternative, we used an expanded PD (aa 1 to 153)-GFP protein as a probe. Signals were most intense on the spots of cardiolipin (CL), phosphatidylcholine (PC), cholesterol (CHOL), sulfatide (SULF), and sphingomyelin (SM) (Fig. 4B). However, the expanded PD protein did not interact with all of the phosphatidylinositol lipids (Fig. 4B), demonstrating specificity. Among the vIRF-1-interacting lipids, CL was of the most interest because it is enriched in the inner mitochondrial membrane (IMM) and exposed on the OMM upon mitochondrial stress (30). As vIRF-1 is associated with the OMM (6), it is likely that vIRF-1 binds directly to externalized CL via the PD or its expanded domain. To quantitatively characterize the vIRF-1 binding to CL, we employed an ELISA-based assay. The expanded PD (1 to 153) and PD (1 to 75), but not GFP, bound to CL (Fig. 4C); the respective dissociation constant (K_d) of binding by the expanded PD (aa 1 to 153) and PD were 0.6 μM and 1.2 μM (Fig. 4D).

Cholesterol is a major constituent of lipid rafts and comprises 1 to 4% of mitochondrial membrane lipids. Thus, it is likely that cholesterol may be involved in stable mDRM targeting of vIRF-1 in addition to CL. By inspection of the expanded PD sequences, we identified the cholesterol recognition/interaction amino acid consensus (CRAC; L/V-X₁₋₅-Y-X₁₋₅-K/R) at the region encompassing L138 to R146 (Fig. 4E). To test if the putative cholesterol-binding domain (CBD) of vIRF-1 is involved in mDRM targeting, we generated a CBD mutant (CBD^X), in which key residues were replaced with alanine (Fig. 4E). As expected, our mDRM targeting assay showed that the mDRM targeting of the expanded PD (1 to 151) was reduced when the CBD was mutated (Fig. 4E). Consistent with this finding, methyl- β -cyclodextrin (M β CD)-mediated cholesterol depletion from the mitochondria exhibited the same effect as the CBD mutation (data not shown). Taken together, these

results suggest that vIRF-1 interactions with membrane lipids such as CL and cholesterol are involved in mDRM targeting and/or anchoring.

MAVS promotes mDRM targeting of vIRF-1. We observed that mDRM targeting of MAVS as well as vIRF-1 was induced during HHV-8 lytic replication (Fig. 5A). MAVS is also known to localize to other subcellular organelles, such as endoplasmic reticulum (ER), mitochondrion-associated ER membranes (MAM), and peroxisome, which are often contaminated in the preparation of mitochondria. However, no significant amounts of their respective markers, calregulin, ACSL4, and catalase, were detected in our mitochondrial preparation (Fig. 5A). In addition, mitochondrial soluble factor cytochrome *c* was not detected in mDRM, while VDAC1 was detected in mDRM (Fig. 5A). These results indicate specific targeting of MAVS to mDRM. From these findings, we postulated that MAVS might have an effect on vIRF-1 targeting to mDRM. To investigate if MAVS is required for HHV-8 replication-induced mDRM targeting of vIRF-1, we lentivirally transduced control and MAVS shRNAs into BCBL-1 TRE:RTA cells, followed by treatment with DOX to reactivate HHV-8 for 1 day. Indeed, both basal and HHV-8-replication-induced mDRM targeting of vIRF-1 were diminished in MAVS-depleted BCBL-1 cells (Fig. 5B). To explore this further, we generated control and MAVS knockout (KO) 293T cells using CRISPR/Cas9 technology (see Materials and Methods) and tested for mDRM targeting of vIRF-1 following MAVS-activating vesicular stomatitis virus (VSV) infection of these cells. While VSV infection induced mDRM targeting of vIRF-1 in control 293T cells, mDRM targeting of vIRF-1 was not induced by VSV infection in MAVS KO cells (Fig. 5C). It is noteworthy that the basal level of vIRF-1 in the mDRM was also decreased in MAVS KO 293T cells, as in BCBL-1 cells. Collectively, these results suggest

FIG 3 vIRF-1 PD has sequence similarity with cellular mDRM-targeting proteins. (A) Two regions (I and II) within vIRF-1 PD have sequence similarity to the latter part of PINK1 mitochondrial targeting sequences (MTS). Identical and homologous amino acids are highlighted with dark blue and pale blue, respectively. TM, transmembrane domain. (B) Identification of the mDRM-targeting region of PINK1. An mDRM targeting assay was performed with the mitochondria isolated from 293T cells transfected with full-length PINK1 (FL) and a mutant (Δ32-60) lacking residues 32 to 60. “ΔN” indicates a natural proteolytic product of PINK1. MAVS was used as a loading control. (C) Functional equivalence of vIRF-1 PD and PINK1 MTS with respect to mDRM targeting. An mDRM targeting assay was performed with 293T cells transfected with vIRF-1 ΔPD or MTS-tagged ΔPD constructs. The indicated MTSs were fused to the N-terminal region of vIRF-1 ΔPD. FLOT1, cytochrome c, and TOM20 were used as loading controls. (D) Alignment of the peptide sequences containing the GxRP motifs of vIRF-1, MAVS, and PINK1. The GxRP sequences among the proteins are highlighted in red. The sequences conserved at the N-terminal region of the GxRP motifs of PINK1 and MAVS are highlighted in purple. (E) The requirement of the GxRP motif for vIRF-1 binding to the mitochondria. *In vitro* mitochondria pulldown assay was performed with purified recombinant GFP, PD-GFP, and PD (GxRP^X)-GFP proteins. ‘GxRP^X’ indicates the mutation of the GxRP motif to AxAA. Recombinant proteins (10% of input) and precipitated complexes were resolved by SDS-PAGE and immunoblotted with antibodies to GFP or heat shock protein 60 (HSP60), a mitochondrial protein. T, total cell extracts, S, supernatant, P, pellet; Mito, mitochondria.

that MAVS plays a crucial role in vIRF-1 recruitment to mDRM upon virus infection and replication.

Next, we determined if the PD domain is essential for MAVS-induced mDRM targeting of vIRF-1. The mDRM targeting assay was conducted with 293T cells transfected with full-length and PD deletion versions of vIRF-1 (vIRF-1 FL and vIRF-1 ΔPD) in the presence or absence of MAVS. Overall levels of vIRF-1 FL and vIRF-1 ΔPD were increased by coexpression of MAVS in 293T cells (Fig. 5D). Thus, we determined the relative levels of the vIRF-1 proteins in the mitochondrial fractions by normalizing by total vIRF-1 content (see the graph in Fig. 5D). Indeed, mDRM and general mitochondrial targeting of vIRF-1 were enhanced by MAVS (Fig. 5D). However, mDRM targeting of vIRF-1 ΔPD was only marginally increased by MAVS compared to targeting of vIRF-1 FL (Fig. 5D). In this experiment, HSP60 was used as a loading control for total cell extracts and mitochondrial soluble fractions. These results indicate that the PD domain plays an important role in MAVS-induced vIRF-1 targeting to mDRM.

vIRF-1 targeting to mDRM is dependent on mitochondrial reactive oxygen species and CL externalization induced by MAVS.

Because MAVS has been known to localize to peroxisome and MAM upon virus infection (31, 32), we investigated if vIRF-1 localizes to these subcellular organelles upon virus infection. HeLa cells were transfected with vIRF-1 and 24 h later were infected with VSV at an MOI of 5 for 6 h, and vIRF-1 was coimmunostained with ACSL4 for MAM or catalase for peroxisome together with TOM20 for mitochondria (Fig. 6A and B). Colocalization of vIRF-1 with the mitochondria was significantly enhanced by VSV infection, with little or no colocalization with peroxisome or MAM markers. These results suggest that the mitochondrion is a main subcellular organelle to which vIRF-1 translocates upon virus infection.

We next sought to determine the molecular mechanism underlying how MAVS drives vIRF-1 specifically to the mitochondria. Recently, it was reported that MAVS could induce production of mitochondrial reactive oxygen species (mROS) (33). Together with the recent finding that CL is externalized by mitochondrial oxidative stress (30), we postulated that MAVS may induce CL externalization through mROS production and thereby recruit vIRF-1 to CL exposed on the mitochondria. First, using a mitochondrial superoxide indicator, MitoSOX red, we observed that

FIG 4 vIRF-1 binds to membrane lipids. (A) Colloidal blue staining of purified recombinant GFP and GFP-fused vIRF-1 proteins. (B) Protein lipid overlay assay. Membrane lipid strips were probed with 1 μ g/ml of recombinant GFP or GFP-fused vIRF-1 expanded PD (aa 1 to 153)-GFP proteins and immunoblotted with anti-GFP antibody. TG, triacylglycerol; DAG, diacylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol; CHOL, cholesterol; SM, sphingomyelin; SULF, sulfatide. (C) ELISA-based analysis of CL binding of vIRF-1 PD. Recombinant GFP or GFP-fused vIRF-1 PD or expanded PD (1 to 153) proteins (2 μ M) were added to CL immobilized on a 96-well plate, and captured proteins were detected by colorimetric reaction (absorbance at 450 nm) using HRP-conjugated GFP antibody and TMB ELISA substrate. (D) A CL-coated plate was incubated with the indicated concentrations of recombinant vIRF-1-GFP fusion proteins. The relative amount of protein bound to CL was determined by calculating $1 - (B_{\max} - B/B_{\max} - B_{\min})$, where B is absorbance at 450 nm, as a percentage and fitted to sigmoidal curve. (E) Involvement of a putative cholesterol-binding domain (CBD) of vIRF-1 in stable targeting to mDRM. A putative CBD motif corresponding to the cholesterol recognition amino acid consensus (CRAC; L/V-(X)₍₁₋₅₎-Y-(X)₍₁₋₅₎-R/K) was identified in the expanded PD region, and the consensus sequences were substituted for alanine (CBD^x). An mDRM targeting assay was performed with 293T cells transfected with constructs carrying GFP-fused vIRF-1 expanded PD (1 to 151) with intact or mutated CBD. MAVS was used as loading control. T, total cell extracts; S, supernatant; P, pellet; Mito, mitochondria.

MAVS was indeed able to promote mROS production, which was abated with an mROS scavenger, MitoTEMPO (Fig. 6C). Furthermore, MitoTEMPO and another mROS scavenger, MitoQ, reduced, by about 45%, vIRF-1 targeting to mDRM induced by MAVS and HHV-8 reactivation (Fig. 6D and E, respectively). As a control, vIRF-1 itself did not produce superoxide in mitochondria and had no effect on MAVS-induced mROS production (Fig. 6F). These results indicate that MAVS- or virus replication-induced mDRM targeting of vIRF-1 is likely mediated by mROS. Next, we developed a method by which exposed CL in the OMM can be assessed quantitatively by determining the level of human anti-CL antibody bound to the mitochondria using SDS-PAGE and immunoblotting (Fig. 7A). As expected, MAVS overexpression in 293T cells increased CL exposure on the mitochondria to up to 70% of the control (Fig. 7B). Similarly, HHV-8 reactivation enhanced CL exposure on the mitochondria, but MitoQ inhibited CL exposure (Fig. 7C). In summary, our results suggest that

HHV-8 replication-induced mDRM targeting of vIRF-1 could be mediated by MAVS-induced mROS production and CL externalization.

vIRF-1 interacts with MAVS and inhibits MAVS aggregation. Recently, it was reported that MAVS may play an inhibitory role in HHV-8 productive replication (15). However, we previously showed that vIRF-1 has an opposite effect, that is, promotion of HHV-8 productive replication (7). Combined with our present data that MAVS promotes vIRF-1 targeting to mDRM during HHV-8 replication (Fig. 5), we postulated that vIRF-1 might associate with MAVS in mDRM and downregulate MAVS-mediated antiviral signaling during virus replication. To test this hypothesis, we first performed coimmunoprecipitations with total cell extracts of resting and reactivated BCBL-1 cells. Indeed, MAVS was detected in vIRF-1 immunoprecipitates (IPs) derived from reactivated BCBL-1 cells, but not in control IgG (nIgG) IPs (Fig. 8A), indicating that vIRF-1 could interact with MAVS upon HHV-8

FIG 5 MAVS is required for mDRM targeting of vIRF-1. (A) MAVS targeting to mDRM during HHV-8 lytic replication. The mitochondria (Mito) of BCBL-1 TRE:RTA cells incubated with or without 1 μ g/ml of doxycycline (DOX) for 2 days were isolated on a self-generating Percoll gradient. Purified mitochondria were incubated in TNE buffer containing 1% Triton X-100 on ice for 30 min, centrifuged into the supernatant (S) and the pellet (P), and immunoblotted with the primary antibodies to vIRF-1, MAVS, and subcellular markers: calregulin for endoplasmic reticulum (ER), acetyl coenzyme A (acetyl-CoA) synthetase long-chain family member 4 (ACSL4) for mitochondria-associated ER membranes (MAM), catalase for peroxisome, and VDAC1 and cytochrome *c* for mitochondria. Twenty-fold excesses of mitochondrial extracts over total cell extract (T) were loaded onto gels to achieve near normalization. (B) MAVS requirement for HHV-8 replication-induced mDRM targeting of vIRF-1. An mDRM targeting assay was performed with the control and MAVS-depleted BCBL-1 TRE:RTA cells treated with DOX for 24 h. Combined MAVS shRNAs (1 and 2) were lentivirally transduced into the cells for 2 days before DOX treatment. Relative band intensities of vIRF-1 in mDRM were determined by fold intensity compared to control (sh-Cont, no DOX). (C) MAVS requirement for VSV infection-induced mDRM targeting of vIRF-1. An mDRM targeting assay was performed in MAVS^{+/+} and MAVS^{-/-} 293T cells transfected with vIRF-1 and infected with VSV at an MOI of 1 for 24 h. Relative band intensities of vIRF-1 in mDRM were determined by fold intensity compared to the control (MAVS^{+/+}, no VSV infection). (D) Requirement of the PD domain for MAVS-induced vIRF-1 targeting to mDRM. An mDRM targeting assay was performed with 293T cells transfected with the indicated vIRF-1 constructions, including full-length (FL) and Δ PD versions together with or without MAVS. The relative band intensities of mitochondrial vIRF-1 in the detergent soluble (S) and insoluble (P) fractions normalized by total vIRF-1 (T) are depicted in the stacked-column graph. FLOT1 and HSP60 were used as loading controls.

replication. Consistent with this finding, immunostaining revealed partial colocalization of MAVS with vIRF-1 in reactivated BCBL-1 cells (Fig. 8B). In addition, vIRF-1 interacted with MAVS in the DRM fraction of transfected 293T cells (Fig. 8C), ruling out the possible requirement for other viral proteins in the interaction. It is noteworthy that overexpression of MAVS in 293T cells induced its smeared MAVS-specific bands in the DRM fraction; these bands may be aggregated and/or polyubiquitinated MAVS that are known to be important for MAVS activation and function. The smeared band formation was largely inhibited in the presence of vIRF-1 (Fig. 8C).

To determine if vIRF-1 can inhibit MAVS aggregation, we performed semidenaturing detergent agarose gel electrophoresis (SDD-AGE), a technique for detecting large protein polymers, such as prions and amyloids (34). Indeed, vIRF-1 diminished the formation of large MAVS aggregates in the mitochondria, while MAVS expression remained constant in the mitochondria and total cell extracts (Fig. 8D). Moreover, we observed that a fraction (15%) of the vIRF-1-positive (+) BCBL-1 cells at 24 h postinduction exhibited a lower intensity of MAVS fluorescence or less MAVS aggregation than did neighboring vIRF-1-negative (-) cells (Fig. 8E). Furthermore, this population was increased more

FIG 6 vIRF-1 targeting to mDRM is promoted by mitochondrial reactive oxygen species. (A and B) Specific targeting of vIRF-1 to the mitochondria after virus infection. HeLa cells were transiently transfected with vIRF-1-Flag for 24 h, infected with VSV at an MOI of 5 for 6 h, and immunostained with rat anti-Flag (L5)-Alexa Fluor 647, mouse anti-TOM20-Alexa Fluor 488, and rabbit anti-catalase-Alexa Fluor 555 (A) or ACSL4-Alexa Fluor 555 (B) antibodies. For comparative analysis with TOM20 (green), pseudocolor images of catalase (green) and ACSL4 (green) were generated using Element software. The images in rectangles with white line are enlarged and shown in the next images. (C) MAVS production of mitochondrial reactive oxygen species (mROS). 293T cells were transfected with or without MAVS for 24 h and then incubated with 5 μ M MitoSOX red reagent in HBSS buffer for 10 min. After fixation with chilled methanol, the cells were immunostained with anti-Flag antibody (green) and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). (D) Role of mROS in MAVS-induced mDRM targeting of vIRF-1. 293T cells were transfected with vIRF-1 and/or MAVS and 12 h later treated with mROS scavengers, including 1 μ M MitoQ and 10 μ M MitoTEMPO, for 12 h. The mDRM fractions and total cell extracts were immunoblotted with the indicated antibodies. Relative band intensities of vIRF-1 in mDRM were calculated by normalization with the band intensity of corresponding MAVS and are shown under the top blot. (E) Role of mROS for HHV-8 replication-induced mDRM targeting of vIRF-1. BCBL-1 TRE:RTA cells were incubated with or without DOX in the presence or absence of 1 μ M MitoQ for 1 day. The mDRM fractions and total cell extracts were immunoblotted with the indicated antibodies. Relative band intensities of vIRF-1 in mDRM were calculated by normalization with the band intensity of corresponding FLOT1 and noted under the top blot. (F) Absence of effect of vIRF-1 on basal and MAVS-induced mROS production. 293T cells were transfected with vIRF-1 with or without MAVS for 24 h and incubated with MitoSOX red as described above. The cells were immunostained with anti-Flag (green) and vIRF-1 (purple), and nuclei were counterstained with DAPI.

than 2-fold (15% to 34%) after 48 h of DOX treatment, implying that MAVS is downregulated during HHV-8 replication, possibly by vIRF-1-mediated inhibition of MAVS aggregation. A region of the MAVS protein termed the caspase activation and recruitment domain (CARD) is known to be involved in the formation of active MAVS aggregates (24). Our coimmunoprecipitation experiment revealed that the interaction of vIRF-1 with MAVS was significantly

reduced when the CARD domain (aa 10 to 77), but not the PD domain (aa 103 to 153), was deleted (Fig. 8F), indicating that the CARD domain of MAVS could mediate interaction with vIRF-1. This result suggests the possibility that vIRF-1 prevents the formation of MAVS aggregates by masking the CARD domain.

vIRF-1 blocks MAVS-induced antiviral signaling. MAVS aggregation is believed to be essential for the activation of MAVS-

FIG 7 CL externalization is promoted by MAVS or HHV-8 replication. (A) Principle of externalized CL assay using human anti-CL antibody. Mitochondria isolated from the sample cells were incubated with anti-CL antibody (0.5 μ l) on ice for 30 min, washed, resolved by SDS-PAGE, and immunoblotted with goat anti-human IgG (α -hIgG). Hc, heavy chain; Lc, light chain. (B) MAVS-induced CL externalization. The externalized CL assay was performed with the mitochondria isolated from 293T cells transfected with or without MAVS for 24 h. One tenth of the input (α -CL) was loaded on the gel. HSP60 was used to examine the level of precipitated mitochondria. (C) MitoQ inhibition of HHV-8 replication-induced CL externalization. The mitochondria isolated from BCBL-1 TRE:RTA cells incubated with or without DOX in the absence or presence of MitoQ for 24 h were subjected to the externalized-CL assay. Normalized band intensities are depicted in the graph. Representative gel images are shown and the data in the graph indicate means $\pm$ SD from three independent experiments.

mediated antiviral signaling that leads to the production of type I IFNs and apoptosis. Thus, we tested if vIRF-1 could inhibit MAVS-induced IFN- β gene expression using a luciferase reporter assay. Previously, vIRF-1 was reported to interfere with IFN- β transactivation by cellular IRFs, including IRF-1 and IRF-3, by blocking promoter recruitment of the CBP/p300 coactivators (35, 36). Therefore, to rule out the nuclear role of vIRF-1, we used the nuclear localization signal (NLS)-mutated version of vIRF-1 (NLS^X) (Fig. 9A), shown previously to be completely inactive in inhibition of p53- or SMAD3-mediated transactivation (6). The vIRF-1 NLS^X variant remained active in the inhibition of MAVS-induced IFN- β transactivation, albeit to a lesser extent than wild-type vIRF-1 (Fig. 9B), indicating a nucleus-independent mechanism of activity consistent with MAVS inhibition. Similarly, vIRF-1 and vIRF-1 NLS^X could inhibit the IFN- β transactivation by a constitutively active form of RIG-I (Δ RIG-I) that leads to MAVS activation (Fig. 9C). We also examined if vIRF-1 inhibits MAVS-induced apoptosis. Using the CellTiter-Glo luminescent cell viability assay, we found that vIRF-1 and the NLS^X variant inhibited MAVS-induced cell death (Fig. 9D). Interestingly, vIRF-1 NLS^X had a more protective effect than wild-type vIRF-1, contrary to their relative inhibitory effects on IFN- β transactivation. Considering that the total expression levels of the vIRF-1 wild type and NLS^X variant are comparable (Fig. 9E), this might be due to the relatively high concentration of vIRF-1 NLS^X in the cytoplasm, where the MAVS-mediated apoptotic signaling occurs. Furthermore, consistent with the results of the cell viability assay, vIRF-1 NLS^X was more effective than vIRF-1 at inhibiting MAVS-mediated apoptotic signaling, as measured by JNK phosphorylation and PARP1 cleavage, a marker of apoptosis (Fig. 9E). An HHV-8 lytic antiapoptotic protein, vBcl-2, was not as effective as vIRF-1 for inhibition of MAVS-mediated cell death and apop-

totic signaling (Fig. 9D and E), suggesting specificity of this antiapoptotic role of vIRF-1 in HHV-8 productive replication. Consistent with anti-MAVS activity of vIRF-1, vIRF-1 could inhibit Δ RIG-I-induced JNK phosphorylation and PARP1 cleavage (Fig. 9F). Taken together, these results suggest that vIRF-1 is a novel viral inhibitor of RIG-I/MAVS-mediated antiviral signaling.

vIRF-1 targeting to mDRM is important for HHV-8 productive replication. To address the biological significance of our findings, we examined the effect of mDRM targeting by vIRF-1 on HHV-8 productive replication and associated cell viability. First, we generated a single lentiviral vector specifying both vIRF-1 or control shRNAs and vIRF-1^R variants, including full-length and Δ PD vIRF-1 (Fig. 10A), which have third codon position silent mutations refractory to the vIRF-1 shRNA. Indeed, Flag-tagged vIRF-1^R variants but not Myc-tagged vIRF-1 wild-type and endogenous vIRF-1 were resistant to depletion by vIRF-1 shRNA in 293T cells and reactivated BCBL-1 cells, respectively (Fig. 10B and D). We observed that vIRF-1 depletion reduced HHV-8 productive replication (Fig. 10C) and increased PARP cleavage (Fig. 10D) in reactivated BCBL-1 cells. The effects were reversed substantially by reconstitution with vIRF-1^R, but vIRF-1^R Δ PD activities were marginal, indicating that mDRM targeting of vIRF-1 via the PD domain is important for promoting HHV-8 productive replication and blocking apoptosis. We observed no effect of the PD deletion on vIRF-1 inhibition of p53- and SMAD3-mediated transactivations (data not shown), suggesting that the effects of PD deletion were related to deficient MAVS inhibition specifically.

DISCUSSION

In this study, we obtained evidence that MAVS activation by virus infection and replication promotes mDRM targeting of vIRF-1,

FIG 8 vIRF-1 interacts with MAVS and inhibits MAVS aggregation. (A) HHV-8 replication-induced vIRF-1–MAVS interaction. BCBL-1 TRE:RTA cells were treated with 1 μ g/ml of DOX or mock treated for 1 day, and total cell extracts were used for immunoprecipitation with anti-vIRF-1 antibody or normal rabbit immunoglobulin (nIgG). The immunoprecipitates and lysates were immunoblotted with the indicated primary antibodies. The arrow indicates MAVS that coimmunoprecipitated with vIRF-1. The asterisks indicate heavy chain of IgG used for immunoprecipitation. (B) Colocalization of vIRF-1 with MAVS in lytic BCBL-1 cells. BCBL-1 TRE:RTA cells were treated with DOX for 24 h and immunostained with mouse anti-MAVS and rabbit anti-vIRF-1 antibodies along with nuclear counterstaining with DAPI. The arrows indicate regions of colocalization. (C) Intracellular interaction of vIRF-1 with MAVS. The Flag-tagged immunoprecipitates and homogenates of the total DRM fractions derived from 293T cells transfected with Flag-MAVS and/or Myc-vIRF-1 were immunoblotted with anti-Myc or Flag antibodies. (D) vIRF-1 inhibition of MAVS aggregation. The total cell extracts and mitochondrial fractions derived from 293T cells transfected with MAVS alone or together with vIRF-1 were resolved by SDD-AGE and SDS-PAGE and immunoblotted with the indicated antibodies. (E) Altered fluorescence of MAVS in vIRF-1-expressing BCBL-1 cells. BCBL-1 TRE:RTA cells were treated with DOX for 0, 24, and 48 h and immunostained with antibodies to vIRF-1 and MAVS. Nuclei were counterstained with DAPI. Example images showing reduced MAVS intensity in vIRF-1-positive (+) cells (white circle) at 24 and 48 h are shown. The MAVS fluorescent intensity and/or aggregates (big speckles) of 150 vIRF-1 (+) cells from 50 randomly selected microscopic fields (60 $\times$)

FIG 9 vIRF-1 inhibits MAVS-mediated antiviral signalings. (A) vIRF-1's nuclear localization signal (NLS) and its mutation (NLS^x). (B and C) vIRF-1 inhibition of MAVS-induced and a constitutively active RIG-I (Δ RIG-I)-induced IFN- β expression. IFN- β -Luc reporter assays were performed with 293T cells transfected with the indicated constructs for 24 h. Results are presented as means $\pm$ standard deviations in triplicate. *, $P < 0.05$; **, $P < 0.01$. (D) vIRF-1 inhibition of MAVS-induced cell death. The viability of 293 cells was assessed at 24 h and 48 h after transfection with MAVS and/or vIRF-1 using a CellTiter-Glo luminescence assay kit. Representative results are presented as means $\pm$ standard deviations. (E) The total cell extracts collected at 24 h for panel D were immunoblotted with the indicated antibodies, including anti-phospho-JNK1/2 (T183/Y185) antibody. (F) vIRF-1 inhibition of Δ RIG-I-induced apoptotic signaling. 293 cells were transfected with GFP or Δ RIG-I-GFP in the presence and absence of vIRF-1 for 24 h and immunoblotted with the indicated antibodies.

which, in turn, inhibits MAVS-mediated antiviral signaling pathways leading to type I IFN gene expression and apoptosis. To our knowledge, this is the first report of negative-feedback regulation of MAVS by a viral gene product, although some viral proteins directly inhibit MAVS by inducing its cleavage or degradation. For example, viral proteases such as hepatitis C virus NS3/4A, hepatitis A virus 3ABC, and enterovirus 71 protease 2A^{pro} cleave MAVS to dislocate the functional domain of MAVS from the mitochondria (13, 37, 38). Hepatitis B virus X protein promotes the protea-

somal degradation of MAVS (39). However, there is no previous evidence that viral gene products are recruited by MAVS to the mitochondria and mediate negative-feedback control during virus replication.

MAVS recruitment of vIRF-1 to mDRM relies on the N-terminal region of vIRF-1, which contains motifs that mediate binding to membrane lipids, including CL. More than 70% of the PD region (residues 1 to 75) consists of proline (15%), glycine (17%), alanine (12%), serine (13%), and charged amino acids (21%),

were compared with those of the neighboring vIRF-1 negative (-) cells, and three types of population are grouped and depicted in the pie diagrams: red, stronger MAVS signals and/or intense MAVS aggregates in vIRF-1 (+) cells than in neighbor vIRF-1 (-) cells [vIRF-1 (+) > vIRF-1 (-)]; green, the same as neighbor vIRF-1 (-) cells [vIRF-1 (+) = vIRF-1 (-)]; and blue, less MAVS signal in vIRF-1 (+) cells than in neighbor vIRF-1 (-) cells [vIRF-1 (+) < vIRF-1 (-)]. (F) Mapping of vIRF-1 binding region of MAVS. A coimmunoprecipitation assay was performed with RIPA buffer-soluble extracts derived from 293T MAVS^{-/-} cells transfected with full-length, Δ CARD (lacking aa 10 to 77), or Δ PD (lacking aa 103 to 153) versions of Flag-MAVS together with vIRF-1.

FIG 10 vIRF-1 PD is important for HHV-8 replication. (A) Diagram of a single lentiviral vector encoding control or vIRF-1 shRNAs together with vIRF-1^R variants resistant to vIRF-1 shRNA. (B) Evaluation of vIRF-1 shRNA and vIRF-1^R variants resistant to vIRF-1 shRNA. 293T cells were cotransfected with control or vIRF-1 shRNAs along with Flag-vIRF-1^R (full-length or ΔPD) and Myc-vIRF-1, which is sensitive to vIRF-1 shRNA. Total cell extracts were immunoblotted with anti-Flag, Myc, and β-actin antibodies. (C) Contribution of the PD domain to vIRF-1 promotion of HHV-8 productive replication in PEL cells. BCBL-1 TRE:RTA cells were lentivirally transduced with control or vIRF-1 shRNAs together with vIRF-1^R variants. Encapsidated HHV-8 virions from the culture media of the transduced BCBL-1 TRE:RTA cells treated or untreated with DOX for 2 days were collected and subjected to qPCR-based viral genome copy number analysis. The data in the graph are means $\pm$ SD from triplicate samples. (D) Verification of vIRF-1 depletion and reconstitution in BCBL-1 TRE:RTA cells. Total extracts collected from the cells for panel C were immunoblotted with the indicated antibodies to PARP, vIRF-1, Flag, and LDH.

which are residues found abundantly in intrinsically disordered proteins (IDPs) (40), defined by the lack of a single, stable tertiary structure under physiological conditions (41). Indeed, bioinformatics tools such as FoldIndex (42) and i-TASSER (43) predict that the PD is likely an unfolded domain (data not shown). IDPs and IDP domains are involved in a variety of functions in the cell, including subcellular localization of proteins (44). For example, the N-terminal IDP domain of a yeast protein, Mgm101p, can act as an MTS (45). Thus, the disordered PD domain of vIRF-1 could serve as an MTS by recognizing CL exposed on the OMM. Furthermore, as shown in Fig. 4, our PLO assay provided evidence that the expanded PD domain interacts directly with other membrane lipids, such as cholesterol, sphingomyelin, and sulfatide, which are constituents of lipid rafts and found in the mitochondrial membranes (46–48), suggesting that stable mDRM targeting of vIRF-1 could be mediated, in a cooperative manner, by multiple regions. Consistent with this notion, it is likely that MAVS targeting to mDRM relies on multiple domains, including the transmembrane domain and the GxRP motif (29).

Despite disagreement on the presence of mDRM (49), there is compelling experimental evidence supporting their existence. The evidence includes (i) proteomic detection of mitochondrial proteins in lipid raft-enriched membranes isolated from internal organelles (50–53) and (ii) alteration of mitochondrial structure and bioenergetics with the use of reagents disrupting lipid rafts such as MβCD or edelfosine (54, 55). mDRM has been proposed to play a

role in the regulation of apoptosis via GD3 trafficking (56) and in recruitment of proapoptotic proteins, including tBid and caspase-8 (53). In addition, key proteins involved in mitochondrial quality control and innate immune responses, such as PINK1 and MAVS, respectively, are translocated to mDRM upon stress (28, 57). Thus, vIRF-1 targeting to mDRM is likely to be involved in the regulation of multiple mitochondrial functions as well as MAVS-mediated antiviral signaling pathways. In fact, we previously found that vIRF-1 binds to tBid and inhibits tBid-mediated cytochrome *c* release from mitochondria (6).

Overall, our findings suggest that mDRM targeting of vIRF-1 is a key event for the feedback inhibition of MAVS aggregation and its antiviral signaling. Although the molecular mechanism by which MAVS is activated by HHV-8 replication is unknown, it is clear that MAVS plays an inhibitory role in HHV-8 replication (15). In addition, we have identified structural determinants and the molecular mechanisms by which vIRF-1 is recruited to mDRM by MAVS and, in turn, inhibits MAVS aggregation during HHV-8 replication. These results will extend our understanding of viral mechanisms countering MAVS-activated innate immunity and may provide a basis for the development of novel antiviral agents.

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