Toll-like Receptor-dependent and -Independent Viperin Gene Expression and Counter-regulation by PRDI-binding Factor-1/BLIMP1

Received for publication, May 11, 2006, and in revised form, July 12, 2006. Published, JBC Papers in Press, July 18, 2006, DOI 10.1074/jbc.M604516200

Martina Severa, Eliana M. Coccia, and Katherine A. Fitzgerald

From the Division of Infectious Disease and Immunology, Department of Medicine, The University of Massachusetts Medical School, Worcester, Massachusetts 01605 and the Department of Infectious, Parasitic, and Immuno-Mediated Diseases, Istituto Superiore di Sanita, Viale Regina Elena 299, 00161 Rome, Italy

Here we identify Viperin as a highly inducible gene in response to lipopolysaccharide (LPS), double-stranded RNA (poly(I-C)) or Sendai virus (SV). The only known function of Viperin relates to its ability to inhibit human Cytomegalovirus replication. Very little data are available on the regulation of this gene. In silico analysis of the promoter identified two interferon (IFN)-stimulated response elements (ISRE), which in other genes bind IRF3 or the IFN-stimulated gene factor-3 (ISGF3) complex. LPS and poly(I-C) induce very high levels of Viperin in wild type cells but not in cells deficient in TRIF, TBK1, IRF3, or the type I IFNα/βR. SV-induced Viperin gene expression was mediated independently of Toll-like receptor (TLR) signaling by retinoic acid-inducible gene (RIG-I) and the downstream adapter, mitochondrial anti-viral signaling (MAVS). Virus-induced Viperin expression was not attenuated in macrophages deficient in either TBK1 or IKKe alone. Moreover, IRF3-deficient, but not IFNα/βR deficient, macrophages still induced Viperin in response to SV. Promoter reporter studies combined with DNA immunoprecipitation assays identified the ISGF3 complex as the key regulator of Viperin gene expression. Moreover, positive regulatory domain I-binding factor 1 (PRDI-BF1, also called BLIMP1) binds the ISRE sites and competes with ISGF3 binding in a virus inducible manner to inhibit Viperin transcription. Collectively, these studies identify Viperin as a tightly regulated ISGF3 target gene, which is counter-regulated by PRDI-BF1.

The key to a successful anti-viral response relies on early detection of virus infection, followed by the rapid production of type I IFNs (IFN-α and IFN-β) and the induction of hundreds of interferon-stimulated genes (ISGs), which limit viral replication and spread. Early detection can occur via the recognition of double-stranded RNA (dsRNA), which accumulates in virus-infected cells. Double-stranded RNA is recognized by (at least) two distinct classes of pattern recognition receptors. TLR3 (1) is an endosomally localized membrane bound receptor which upon dsRNA recognition oligomerizes and recruits a Toll-interleukin-1 receptor (TIR) domain containing adapter molecule, TRIF (also called TICAM1). TRIF is one of four adapters involved in TLR signaling, which interacts with TRAF proteins and downstream kinase complexes (reviewed in Ref. 2). Recognition of the Gram-negative bacterial product lipopolysaccharide (LPS) occurs via TLR4 and MD2, which engage all four TIR adapters: MyD88, Mal (TIRAP), TRIF, and TRAM (2).

A second dsRNA sensing system, localized in the cytoplasm, is mediated by the RNA helicases: retinoic acid-inducible gene (Rig-I) (3) and melanoma differentiation associated antigen-5 (Mda-5) (4). These RNA helicases bind dsRNA and activate downstream signaling via caspase recruitment and activation domains (CARD), which engage MAVS (mitochondrial antiviral signaling, also called IPS1, CARDif, or VISA) a CARD domain-containing adapter via homotypic interactions (5–8). Like TRIF, MAVS recruits TRAF proteins and downstream kinases to activate transcription (for a complete review, see Ref. 9). Signaling by TLR3, TLR4, and these helicases leads to the activation of ATF-2-c-Jun, IRF3/IRF7, and NF-κB. In resting cells, NF-κB is bound to the inhibitor protein IκBα, which, after virus infection, becomes phosphorylated by the classical IκB kinase complex (IKKα/β/γ), “marking” IκBα for ubiquitination and subsequent degradation by the proteasome. Similarly, IRF3 becomes phosphorylated, but in this case IKKe (IKKδ) and/or TBK1 (NAK,T2K) (10, 11) mediate this phosphorylation. The ATF-2-c-Jun complex is also rapidly activated by the c-Jun amino-terminal kinase (JNK). ATF-2-c-Jun, IRF3/IRF7, and NF-κB form a multiprotein complex, the “enhanceosome,” on the IFN-β promoter to regulate IFN-B gene expression.

IFN-β signals in an autocrine/paracrine manner via the heterodimeric receptor complex (IFNα/βR) 1 and IFNα/βR2 (12). Each of these receptor subunits engages a member of elements; HCMV, human cytomegalovirus; DC, dendritic cells; Ab, antibody; IL, interleukin; SV, Sendai virus; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; TNF, tumor necrosis factor; TNF, TNF receptor; BM, bone marrow; ko, knockout; MEF, mouse embryonic fibroblast.
the Janus-activated kinase (JAK) family (13, 14). IFNα/βR1 is constitutively associated with tyrosine kinase 2 (TYK2), whereas IFNα/βR2 associates with JAK1 (13–16). Ligand-induced rearrangement and dimerization of the receptor subunits causes the receptor-associated JAKs to become activated by autophosphorylation resulting in the tyrosine phosphorylation and heterodimerization of signal transducer and activator of transcription (STAT) 1 and STAT2, which associate in the cytoplasm with IRF9 (also called p48). The trimeric STAT1/2/IRF9 complex, also called the IFN-stimulated gene factor 3 (ISGF3) complex, translocates to the nucleus and initiates transcription by binding IFN-stimulated response elements (ISRE) in the promoters of multiple ISGs. Although some anti-viral genes can be induced directly by IRF3, the main workhorses of the anti-viral response are the diverse ISGs, many of which are of unknown function (17).

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon-γ-inducible) is an anti-viral gene whose function is still unclear. Viperin was initially identified as a human cytomegalovirus (HCMV)- and IFNγ-inducible protein in fibroblasts (18) and was shown to inhibit productive HCMV infection by down-regulating several HCMV structural proteins critical for viral assembly and maturation. Viperin (known as cig5 in humans, vig1 in mouse, and also RSAD2 in both human and mouse) has also been shown to be induced after infection with vesicular stomatitis virus, Yellow Fever virus, human polyomavirus JC, hepatitis C virus (19–22), or after the lipofection-mediated delivery of bacterial or viral DNA into the cytoplasm (23). The rapid and robust induction of the Viperin gene by a range of different viruses and microbial products such as LPS and DNA suggests that it is an important component of innate immunity to diverse pathogens.

In this study, we have examined the molecular mechanisms regulating Viperin gene expression. We demonstrate that Viperin is induced via IRF3-dependent as well as IRF3-independent pathways all of which rely on type I IFN signaling. The key factor regulating Viperin promoter activity appears to be the ISGF3 complex and not IRF3 itself. Moreover, positive regulatory domain I-binding factor 1 (PRDI-BF1, also called BLIMP1) blocks Viperin expression. Collectively these studies identify Viperin as a highly inducible ISGF3 target gene and define the molecular mechanisms controlling its expression.

**EXPERIMENTAL PROCEDURES**

**Generation of Human Dendritic Cells (DC) and Mouse Macrophages**—DC were generated and their maturation state examined as described (24, 25). Bone marrow-derived macrophages were generated as described (26) and their differentiation state confirmed by staining for F4/80 (Caltag, Burlingame, CA) and CD11b (Pharmingen) using pure Abs or as direct conjugates.

**Mice, Cell Lines, Viruses, and Reagents**—MyD88−/−, TRIF−/−, TRAM−/−, and Mal−/− mice were from S. Akira (Osaka University, Osaka, Japan). IRF3−/− mice were from T. Taniguchi (University of Tokyo, Tokyo, Japan). IFNα/βR−/− mice were from J. Sprent (Scripps Research Institute, San Diego, CA). TBK1−/−/TNFR1−/− mice were from T. Mak and W.-C. Yeh, (University of Toronto, Canada) and IKKe−/− mice were from Millennium Pharmaceuticals (Cambridge, MA). HEK293-TLR2, TLR3 and TLR4/MD2 cells were generated as described (26). Mouse embryonic fibroblasts from BLIMP1−/− mice and MEFs targeted by Cre-recombinase (BLIMP1−/−) were prepared by J. Ye and T. Manaitis (Harvard University, Cambridge, MA) from mice provided by K. Calame (Columbia University). Sendai virus, Cantrell strain was from Charles River Laboratories (Boston, MA). Polynosinic-polycytidylic acid (poly(I-C)) was from Amersham Biosciences. Re-extracted LPS was generated as described (27). IFN-β was from Biogen Inc. (Cambridge, MA). TNF-α and IL1-β were from R&D Systems (Minneapolis, MN), and Pam3cysk4 was from EMD Microcollections (Tuebingen, Germany). Sheep antiserum raised against human leukocyte IFN (28) was used at a dilution 1:100.

**Plasmid Constructs**—The human and mouse Viperin promoters were cloned from human THP-1 and mouse ES cell (129SV) genomic DNA, respectively, and inserted into pGL3-basic. pEF-BOS-RIG-I, Mda-5, and the IFN-β luciferase reporter (p125Luc) construct were from T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (3). pEF-Bos MAVS was cloned from human 293T total cDNA. pEF-BOS-Lgp2 and the NF-κB luciferase reporter were as described previously (29). The ISG54-ISRE was purchased from Stratagene (La Jolla, CA). pCDNA3-FLAG-PRD1-BF1, pCDNA3-FLAG-PRD1-BF1A398−789, and pCMV-FLAG-p65 were from T. Maniatis (Harvard University). pRC-CMV-FLAG-STAT1 and pRC-CMV-FLAG-STAT1-Y701 were from G. Stark (Cleveland Clinic, Cleveland, OH). pCDNA3-HA-IKKβ and pCMV-FLAG-IRF3 were from Paula M. Pitha (John Hopkins University, Baltimore, MD); and pRK-HA-IRF1 was from H-B Shu (National Jewish Medical and Research Center, Denver, CO). pCDNA3-FLAG-IKKε and pCDNA3-FLAG-IKKβ have been described (30). pCDNA3-FLAG-TBK1 was from M. Nakanishi (Nagoya, Japan).

**Reporter Assays**—For reporter assays, HEK293 (seeded 10⁵ cells/ml in 96-well plates) were transfected with 40 ng of the indicated luciferase reporter gene together with 40 ng of thymidine kinase Renilla luciferase reporter gene (Promega, Madison, WI) and the indicated amount of expression plasmids using Genejuice (Novagen, San Diego, CA). Where indicated SV (300 hemagglutination units/ml) or IFN-β (200 pm) were added for 16 h, and luciferase activity was measured as described previously (10). In all experiments, data were normalized for transfection efficiency with Rotylechulus reniformis luciferase.

**Quantitative Real-time PCR**—RNA from human monocyte-derived DC and murine bone marrow macrophages was extracted with RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized, and quantitative RT-PCR analysis was performed on a DNA engine Opticon 2 cycler (MJ Research, Watertown MA) using the SuperScript III two-step qRT-PCR kit with SYBR Green (Invitrogen). The specificity of amplification was assessed for each sample by melting curve analysis. Relative quantification was performed using standard curve analysis. The human quantifications data are presented as a ratio to the GAPDH level.
and the murine ones were normalized with β-actin. The standard errors (95% confidence limits) were calculated using the Student’s t test. All gene expression data are presented as a ratio of gene copy number per 100 copies of GAPDH or β-actin (as indicated) ± S.D.

The following primer pairs have been used. For human: GAPDH, 5′-ACAGTCCATGCATACGTGCC-3′ (forward) and 5′-ACAGTCCATGCATACGTGCC-3′ (reverse); cig5, 5′-GCTTGTGCTGCCCCTTGAGGAA-3′ (forward) and 5′-GCTTGTGCTGCCCCTTGAGGAA-3′ (reverse); cig-5/Viperin gene expression in human DC. A total RNA was extracted from nonstimulated (ns), Pam2Cysk4 (10 nm), poly(dI-dC) (p/C) (100 μg/ml), LPS (100 ng/ml), and TNF-α (50 ng/ml) + IL1-β (50 ng/ml)-treated DC upon 3-h treatment. Viperin and GAPDH mRNA expressions were analyzed by real-time RT-PCR, and data were normalized with GAPDH level of expression.

RESULTS

We first examined cig-5/Viperin gene expression in human monocyte-derived DC in response to various stimuli. DC were treated for 3 h with the synthetic mycobacterial lipoprotein, Pam2Cysk4, poly(dI-dC), and LPS, ligands for TLR2, TLR3, and TLR4, respectively. As seen in Fig. 1A, quantitative real-time RT-PCR measurements demonstrate that both poly(dI-dC) and LPS induced a robust induction of Viperin. In contrast Pam2Csk4 or the mixture of pro-inflammatory cytokines

MgCl2, 300 mM KCl, 0.2 mM EGTA, pH 7, and 25% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and mixture protease inhibitors). The suspensions were clarified by centrifuging at 15,000 × g for 15 min. The nuclear extracts were stored at −20 °C.

DNA Affinity Purification Assay—Biotinylated oligonucleotide ISRE (5′-GTTCATAGGTGGAAAATCGAAACTCTAAC-3′) was annealed with the corresponding antisense oligonucleotide in 1× STE buffer, containing 10 mM Tris-HCl, pH 8, 50 mM NaCl, and 2 mM EDTA. Biotinylated DNA oligonucleotides were mixed with 500 μg of nuclear extracts in 500 μl of binding buffer containing 20 mM Tris-HCl, pH 7.5, 75 mM KCl, 1 mM dithiothreitol, and 5 mg/ml bovine serum albumin in presence of 13% glycerol and 20 μg of poly(dI-dC) and incubated for 25 min at room temperature. Then streptavidin magnetic beads (Promega), washed three times with 400 μl of 1× binding buffer and were added to the reaction mixture and incubated for 30 min at 4 °C and for 10 min at room temperature with mixing by rotation. The beads were collected with a magnet and washed three times with 500 μl of binding buffer. The bound proteins were eluted from the beads by boiling in sample buffer and were resolved on 7.5% SDS-PAGE followed by immunoblotting with the indicated specific Abs: anti-HA-horseradish peroxidase (Roche Applied Science), anti-FLAG-horseradish peroxidase (Sigma), anti-STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PRDI-BF1 (Novus Biologicals Inc., Littleton, CO). The level of the nuclear lysates used in the assay was verified by immunoblotting with the anti-USF2 (Santa Cruz Biotechnology).

Western Blot Analysis—Nuclear cell extracts were separated by 7.5% SDS-PAGE gel and blotted onto nitrocellulose membranes. Blots were incubated with rabbit polyclonal or mouse monoclonal Abs, as requested, against the specified Abs using an ECL system (Amerham Biosciences).

FIGURE 1. Regulation of Viperin gene expression in human DC. A, total RNA was extracted from nonstimulated (ns), Pam2Cysk4 (10 nm), poly(dI-dC) (p/C) (100 μg/ml), LPS (100 ng/ml), and TNF-α (50 ng/ml) + IL1-β (50 ng/ml)-treated DC upon 3-h treatment. Viperin and GAPDH mRNA expressions were analyzed by real-time RT-PCR, and data were normalized with GAPDH level of expression. B, schematic map of ~500 bp of the human Viperin promoter. TSS, transcription starting site. TATAAA is the consensus sequence for the TATA box. C, Viperin reporter activity in HEK-TLR2, HEK-TLR3, and HEK-TLR4/MD2 + TRAM treated with the respective ligands and in HEK cells stimulated with TNF-α + IL1-β and SV.
TNF-α and IL1-β did not induce this gene. Sendai virus (SV) also induced a strong expression of Viperin in DC. This differential inducibility of Viperin prompted us to examine its promoter region for transcription factor-binding sites. Using the transcription factor-binding site prediction program MatInspector (31), a region spanning 1,000 bp upstream of the transcription starting site and 500 bp downstream was analyzed. Several transcription factor-binding sites for IFN-regulatory factors (IRF1, IRF2, IRF3, and IRF4), as well as two ISREs, which can bind IRFs and the IFN-stimulated gene factor-3 (ISGF3) complex, were identified within 500 bp of the transcription starting site. An NF-κB-binding site in close proximity to the transcription starting site was also detected (Fig. 1B). Interestingly, we also found binding sites for PRDI-BF1 (see below).

We next examined the activation of both human and mouse Viperin promoters generated using luciferase-based reporter constructs. HEK293 cells expressing TLR3 induced the human Viperin promoter following treatment with poly(dI-dC). HEK293 cells expressing TLR4, MD2, and the TLR4-specific adapter TRAM also induced this reporter in response to LPS (of note, we did not observe either Viperin or IFNB reporter gene activation without TRAM coexpression). Consistent with our data in human DC, neither the TLR2 ligand nor IL-1β/TNF-α treatment induced the reporters (Fig. 1C). Importantly, these latter treatments strongly induced an NF-κB-driven reporter gene (data not shown). SV infection also resulted in a strong activation of the reporters in the parental cells in the absence of exogenous TLR expression. Similar results were obtained using the murine promoter (data not shown).

We next examined Viperin induction in bone marrow (BM) macrophages derived from MyD88-, Mal-, TRIF-, and TRAM-deficient mice following LPS treatment to probe the adapter molecule requirements for this response. Macrophages from these mice were treated for 3 h with LPS and the level of Viperin mRNA was examined by quantitative RT-PCR (Fig. 2A). Viperin gene expression was completely abrogated in LPS-treated macrophages from TRIF- and TRAM-deficient mice. In contrast, the response in both Mal- or MyD88-deficient mice was not impaired, in fact these two strains showed an even stronger induction compared with C57BL/6 wild type mice highlighting the possible involvement of a MyD88/Mal-dependent repressor of Viperin gene expression (see “Discussion”). These findings support a key role for the TRIF/TRAM pathway in the induction of Viperin following LPS treatment.

We have previously shown that the RNA helicase RIG-I is important in sensing of SV (29). To determine whether RIG-I contributes to SV-induced Viperin gene expression, HEK293 cells were transfected with a reporter gene encoding the human Viperin promoter and cotransfected with an expression vector for RIG-I or Mda-5 (Fig. 2B). Transfection of the full-length RIG-I alone activated the Viperin reporter and this effect was dramatically enhanced upon SV infection. Mda-5 overexpression slightly induced Viperin promoter; however, no enhancement was observed with SV (29). Lgp2, a protein structurally related to RIG-I (32), which seems to be a post-induction feedback inhibitor of RIG-I signaling (29, 33), decreased SV-induced Viperin reporter activity (Fig. 2B). To clearly demonstrate a role for RIG-I we used the human Huh7.5 hepatoma cell line that has a single point mutation within the CARD domain of RIG-I, which impairs its signaling. Consistent with the previous data, the Viperin promoter was induced in the parental Huh7 cell line upon SV stimulation. However, we observed an induction in Huh7.5 upon SV infection only after their complementation with wild type RIG-I (Fig. 2C). Moreover, overexpression of MAVS in HEK293 cells potently activated the Viperin promoter, and this response was enhanced by SV infection (Fig. 2D). SV-induced Viperin reporter activity was blocked by the Hepatitis C virus NS3/4A protease (data now shown), in fact it has been recently shown that NS3/4A abrogates signaling through RIG-I by cleaving MAVS from the mitochondrial membrane (34). Collectively these data demonstrate that RIG-I and MAVS regulate Viperin gene expression in the SV pathway.

We next examined Viperin reporter gene activity upon overexpression of the IKK kinases, which act downstream of TRIF and MAVS to regulate either IRF3 or NF-κB. Overexpression of TBK1 and IKKe but not the related kinase IKKβ induced the reporter gene (Fig. 3A). TBK1, but neither IKKe nor IKKβ, greatly enhanced SV-induced reporter activity suggesting that
FIGURE 3. Viperin gene expression is regulated via the TBK1/IRF3 axis. A, HEK293 cells were cotransfected with vector encoding a reporter gene for Viperin and expression vectors for TBK1, IKKe, or IKKβ. After 8 h, some cells were infected with Sendai virus and Luciferase Assay was then performed. B–D, BM macrophages from C57BL/6, IKKe−/−, or TBK1−/−TNFR1−/− (B) IRF3 (C), or IFNα/βR (D) mice were differentiated and treated with LPS, poly(dI-dC), or SV for 6 h. Total RNA was extracted and the Viperin mRNA quantified by real-time RT-PCR. E, total RNA was isolated from human DC following LPS treatment in presence or absence of antibodies neutralizing type I IFNs. mRNA expression of the Viperin gene was analyzed by real-time RT-PCR. Data were then normalized with GAPDH levels of expression. ns, nonstimulated.

TBK1 may be the main kinase regulating the Viperin response. The lack of induction by IKKβ suggests that the NF-κB site may not be as important. We also compared the induction of Viperin in macrophages from C57BL/6 wild type, IKKe-deficient, and TBK1/TNFRI double ko mice. TBK1-deficient mice are embryonic lethal; however, this lethality can be rescued by crossing these mice onto the TNFR1−/− background. C57BL/6 and TNFR1−/− mice induced equivalent levels of Viperin in response to all stimuli tested (data not shown). The induction of Viperin in response to both LPS and poly(dI-dC) was completely impaired in TBK1/TNFRI ko mice (Fig. 3B). A minor defect was also seen in IKKe-deficient macrophages. In contrast, when SV was examined, the induction of Viperin was intact in both strains. The induction of Viperin in response to LPS and poly(dI-dC) was also completely impaired in macrophages from IRF3-deficient mice (Fig. 3C). Conversely, the virus-induced response in IRF3-deficient macrophages was completely normal.

To determine whether Viperin was a direct IRF3 target gene or if this gene was induced as a result of IFN-β signaling, we examined C57BL/6 and IFNα/βR ko macrophages. Viperin was not induced in response to LPS, poly(dI-dC), or SV in macrophages from IFNα/βR ko (Fig. 3D), suggesting that Viperin induction mainly relies on IFN-β signaling regardless of the stimuli. Pam3CSK4 fails to induce Viperin mRNA expression consistent with a failure of TLR2 ligands to induce IFN-β (35). To further prove that type I IFN production and IFN signaling via IFNα/βR are critical for Viperin gene regulation, we measured Viperin mRNA level by real-time RT-PCR either in unstimulated or LPS-treated DC in the presence or absence of antibodies neutralizing type 1 IFNs (α-IFNs Abs) (Fig. 3E). The endogenous expression of Viperin gene observed in DC upon LPS treatment was completely abrogated when IFNβ signaling was blocked by adding α-IFN Abs.

Since our in silico analysis identified two putative ISREs in the Viperin promoter, and type I IFN target genes are known to be regulated via ISGF3-dependent ISRE binding, we next examined the effect of overexpressing IRF9 and STAT1, which bind the ISRE (36–38). We also analyzed the involvement of IRF1, IRF3, and the NF-κB subunit p65 (Fig. 4A). While p65 failed to induce the Viperin reporter, an NF-κB reporter was strongly induced in response to p65 overexpression (data not shown). IRF1 induced the Viperin reporter gene; however, there was no enhancement by SV under these conditions. The ability of IRF1 to induce the reporter is consistent with a role for IRF1 in mediating the IFN-γ induced Viperin induction (18). IRF3 also induced a very weak Viperin promoter activity, and this response was not dramatically enhanced upon SV infection. Most significantly, when either IRF9 or STAT1 was expressed a clear enhancement was detected upon SV infection (Fig. 4A).

We next performed DNA affinity purification assays to examine ISGF3 complex binding to the ISRE sites. For this purpose, biotinylated oligonucleotides corresponding to the two tandem ISRE sites were incubated with nuclear extracts from HEK293 cells transiently transfected with IRF9 or STAT1 and infected or not with SV. Both IRF9 and Stat1 binding to the ISRE sites could be detected post-virus infection (Fig. 4B). We could also detect endogenous STAT2 binding to this site (data not shown). Equivalent levels of transfected proteins were observed (Fig. 4B, lower panels). Moreover, the overexpression of a STAT1 mutant (STAT1-Y701), which cannot be activated by phosphorylation, dose-dependently blocked virus- and IFNβ-induced Viperin reporter gene induction (Fig. 4C). The STAT1-Y701 mutant did not alter the activity of a virus induced IFNβ or NF-κB reporter gene, as expected.

The in silico analysis of the Viperin gene promoter also led to the identification of two binding sites for the transcriptional repressor PRDI-BF1, in the same region as the binding sites for IRF2 and overlapping IRF3/ISRE sites. We therefore assessed the effect of PRDI-BF1 on the regulation of Viperin gene transcription. Exogenous expression of PRDI-BF1 completely abolished SV-induced Viperin reporter gene activation (Fig. 5A).
Consistent with published data, PRDI-BF1 also blocked SV-induced IFN-β reporter gene expression. Notably, NF-κB activation was unaffected. In addition the induction of Viperin by IFN-β was also dose-dependently inhibited by PRDI-BF1 (Fig. 5B), suggesting that the Viperin repression is not indirect via inhibition of IFN-β production. Consistent with its ability to block IFN-β-induced Viperin activation, PRDI-BF1 binding to the ISRE sites was detected in a virus inducible manner (Fig. 5C). Interestingly, the in silico analysis suggested that the PRDI-BF1 sites overlapped the consensus sites for IRF3, which in turn overlaps in part with one of the two ISRE sites. PRDI-BF1 could also be detected on the IRF3 site (Fig. 5C, upper panel). The virus-induced binding of STAT1 to these sites was dose-dependently inhibited in the presence of increasing amounts of PRDI-BF1 (Fig. 6A). The relative amounts of the proteins present in each cell lysate before pull down was examined by Western blotting (lower panels), and the quantity of the nuclear lysates used in the assay was verified by immunoblottting with the ubiquitously expressed nuclear protein USF-2 (Fig. 6A, bottom panel). Previous published studies have defined a region within PRDI-BF1 between amino acids 331 and 429, which are important for the inhibition of IFN-β gene transcription. To determine whether this same region was critical for suppression of Viperin induction, we examined virus induced reporter gene induction in cells coexpressing either the full-length PRDI-BF1 or a deletion mutant lacking the repression domain. Importantly, while the full-length PRDI-BF1 inhibited Viperin induction, the deletion mutant (PRD-Δ398–789) failed to repress Viperin transcription (Fig. 6B). To define the role of BLIMP1 in the transcriptional regulation of Viperin gene expression in vivo, we measured BLIMP1 mRNA levels in BM macrophages derived from C57BL/6 wild type mice upon LPS and SV treatments at different time points (Fig. 6C). We observed a strong induction of the BLIMP1 gene very early, within 2 h post-infection/treatment. We therefore next employed embryonic fibroblasts (MEFs) lacking BLIMP1 to further understand the relevance of the gene on Viperin gene expression following SV infection. Maximal levels of Viperin
ISGF3- and PRDI-BF1-mediated Viperin Gene Regulation

DISCUSSION

Type I IFN signaling and a number of ISGs are critical for host defense against virus and possibly bacterial infections. A clear component of this host defense mechanism likely involves Viperin, although exactly how Viperin functions in the immune response to agents other than HCMV is unclear at present. In the case of HCMV infection, Viperin inhibits viral assembly and maturation by down-regulating the viral proteins gB, pp28, and pp65 (18). The mechanisms by which Viperin down-regulates the viral proteins are unclear. Viperin is highly conserved between species and contains an MoaA/PQOIII motif, shown to be important for iron-sulfur cluster coordination associated with protein radical formation, and the biosynthesis of cofactors, which may influence anti-microbial defenses (39). Further studies are required to establish the importance of this region and the mechanisms by which Viperin contributes to mammalian host defenses.

Although a number of anti-viral genes can be induced directly by IRF3 without the requirement for type I IFN, this study provides clear evidence that type I IFN signaling and STAT activation are essential for Viperin gene induction, rather that IRF3 by itself. Consistent with their role in the regulation of IFN-β, induction of Viperin upon LPS or poly(dI-dC) treatment was completely dependent on the TBK1/IRF3 axis, in fact Viperin induction was impaired in macrophages deficient in TBK1 or IRF3. However, Viperin was not induced upon LPS treatment in IFNα/βR ko cells, consistent with a model whereby TBK1 and IRF3 control type I IFN production, which then induce Viperin via the IFNα/βR.

The induction of Viperin by the RIG-I/MAVS pathway was not attenuated in macrophages from either TBK1−/−, IKKe−/−, or IRF3−/− deficient mice. This may reflect a functional redundancy between these two kinases in the SV response, since macrophages express both kinases.3 Support for this idea comes from studies in TBK1-deficient embryonic fibroblasts (MEF cells have little or no IKKe expression), which fail to induce Viperin upon SV infection (data not shown). Another possibility is that an additional as yet to be defined kinase may account for the normal induction of Viperin in IRF3-deficient cells.

Although advantageous to the host by inducing genes like Viperin, type I IFNs are not always beneficial. Left uncontrolled excessive IFN production can contribute to autoimmune-like symptoms resembling those of systemic lupus erythematosus, thyroiditis, rheumatoid arthritis, or psoriasis (40). Like other cytokines, therefore, the actions of IFN-α/β and target gene induction must be controlled. Here, we provide evidence that PRDI-BF1/BLIMP1 acts as a negative regulator of Viperin gene expression. We show that PRDI-BF1 inhibits not only virus-induced IFN-β gene expression but also virus-induced and IFNβ-induced Viperin expression. PRDI-BF1 competes with the ISGF3 complex for the ISRE sites. PRDI-BF1 binding to the IRF3 site may also enhance the inhibitory effect on the ISRE sites. PRDI-BF1 and IRF3 control type I IFN production, which then induce Viperin via the IFNα/βR.

3 M. Severa, E. M. Coccia, and K. A. Fitzgerald, unpublished data.
have also detected PRDI-BF1 mRNA induction upon either virus infection or LPS treatment in mouse macrophages and embryonic fibroblasts (data not shown), consistent with PRDI-BF1 acting as a post-induction feedback regulator of gene expression. Indeed, MEFs lacking BLIMP1 show an altered profile of virus-induced Viperin gene induction. These results suggest that Blimp1 is an important negative regulator of Viperin gene regulation in vivo. How the BLIMP1 gene is induced is unclear at present. TLRs may activate PRDI-BF1 as a target of the MyD88-dependent pathway for TLR4 signaling and provide a molecular basis for the elevated induction of Viperin in MyD88-deficient macrophages.

Acknowledgments—We thank Nadege Goutagny and Maria Elena Remoli for valuable discussion and Brian Monks and Zhaohao Jiang for technical assistance.

REFERENCES

1. Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) Nature 413, 790–792.
2. Vogel, S. N., Fitzgerald, K. A., and Fenton, M. J. (2003) Mol. Interw. 3, 446–477.
3. Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004) Nat. Immunol. 5, 730–737.
4. Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S., and Randall, R. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17264–17269.
5. Seth, R. B., Sun, L., Ea, C. K., and Chen, Z. J. (2005) J. Biol. Chem. 280, 34011–34018.
6. Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenbourn, S., and Randall, R. E. (2004) Nature 431, 1167–1172.
7. Xu, L. G., Wang, Y. Y., Han, K. J., Li, L. Y., Zhai, Z., and Shu, H. B. (2005) Mol. Cell 19, 727–740.
8. Peeters, R. T., Liao, S. M., and Maniatis, T. (2000) Mol. Cell 5, 513–522.
9. Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M., and Werner, T. (2005) Bioinformatics 21, 2933–2942.