Essential role of IPS-1 in innate immune responses against RNA viruses

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IFN-β promoter stimulator (IPS)-1 was recently identified as an adapter for retinoic acid–inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (Mda5), which recognize distinct RNA viruses. Here we show the critical role of IPS-1 in antiviral responses in vivo. IPS-1–deficient mice showed severe defects in both RIG-I– and Mda5–mediated induction of type I interferon and inflammatory cytokines and were susceptible to RNA virus infection. RNA virus–induced interferon regulatory factor–3 and nuclear factor κB activation was also impaired in IPS-1–deficient cells. IPS-1, however, was not essential for the responses to either DNA virus or double-stranded B-DNA. Thus, IPS-1 is the sole adapter in both RIG-I and Mda5 signaling that mediates effective responses against a variety of RNA viruses.
The mitochondrial localization of IPS-1 is essential for triggering downstream signaling, indicating a critical link between mitochondria and antiviral immunity. IPS-1 is capable of activating interferon regulatory factor (IRF)-3 and IRF-7. Both IRF-3 and IRF-7 reside in cytoplasm in nonstimulated cells. Upon virus infection, these IRFs are phosphorylated by TANK-binding kinase 1 and inducible inhibitor of kβ kinase to translocate to the nucleus and regulate expression of genes encoding type I IFN (20, 21). IPS-1 also activates NF-κB that controls the expression of genes encoding inflammatory responses via Iκκα- and Iκκβ-mediated phosphorylation and destruction of kBs (22). Fas-associated death domain, receptor-interacting protein 1, and Caspase-8 are suggested to be involved in IPS-1-mediated pathway (23, 24). However, the contribution of IPS-1 in RIG-I– and Mda5-dependent signaling and in antiviral immune responses in vivo remains unclear.

In the present study, we provide evidence for the critical role of IPS-1 in antiviral responses in vivo. IPS-1–deficient mice displayed defective induction of type I IFN and inflammatory cytokines after infection with various RNA viruses and were susceptible to the RNA virus infection. Furthermore, IPS-1–deficient cells were unable to activate NF-κB and IRF-3 in response to NDV. On the other hand, IPS-1 was not essential for the responses to either DNA virus or double-stranded B-form DNA. Collectively, these results demonstrate that IPS-1 is an essential component in both RIG-I– and Mda5–dependent signaling that triggers the host response to infection with various RNA viruses.

RESULTS
Generation of IPS-1–deficient mice
We generated IPS-1–deficient mice by the standard gene targeting. We designed a targeting vector to disrupt two exons harboring the CARDJ-like domain of IPS-1, which is required for signaling (Fig. 1 A). The heterozygosity and homozygosity of obtained mice were verified by Southern blot analysis (Fig. 1 B), and the nullizygosity was confirmed by Northern blot and immunoblot analyses (Fig. 1, C and D). Mutant mice homozygous for the disrupted Ips-1 allele were born at the expected Mendelian ratio and grew healthy in specific pathogen-free conditions. No obvious change in hematopoietic cell development was observed in IPS-1–deficient mice as determined by FACS analysis (unpublished data).

Role of IPS-1 in RIG-I–dependent responses
We first asked whether or not IPS-1 is required for antiviral responses against single-stranded RNA viruses. We used NDV, VSV lacking a variant of M protein (NCP), and SeV with mutated C proteins (Cm) as model viruses for RIG-I–dependent responses (8, 9). Mutant VSV and SeV induce type I IFN in mouse embryonic fibroblast cells (MEFs) caused by an inability to inhibit host IFN responses. Production of IFN-β, IFN-α, and IL-6 after these viruses’ infection was severely impaired in MEFs prepared from IPS-1–deficient mice, as measured by ELISA (Fig. 2 A). Failure of these cytokine’s induction was also observed in peritoneal macrophages (PECs) and granulocyte/macrophage colony-stimulating factor (GM-CSF)–generated bone marrow–derived dendritic cells (GM-DCs) from IPS-1–deficient mice (Fig. S1, available at http://www.jem.org/cgi/content/full/20060792/DC1). Induction of mRNA encoding IFN-α (Ifna), IFN-β (Ifnb), IFN-inducible genes (IP-10 [Cxcl10] and RANTES [Ccl5]), and IL-6 (Il6) in response to these viruses was also impaired in IPS–1–deficient MEFs (Fig. 2 B). We next examined surface expression of costimulatory molecules on DCs, a process required for induction of adaptive immune responses. Surface expression of CD86 after VSV NCP infection was not observed in conventional DCs (Flt3 ligand–generated DCs, Flt3 ligand–generated bone marrow–derived DCs with B220 −) from IPS–1–deficient mice (Fig. 2 C). In contrast, surface expression of CD86 in response to A/D-type CpG DNA (D35), a ligand for TLR9, was normal in IPS–1–deficient DCs (Fig. 2 C). Surface expression of CD40 was also impaired in VSV-infected IPS–1–deficient DCs (unpublished data).
In vitro transcribed dsRNA is shown to induce type I IFN in a manner dependent on RIG-I (9). We next synthesized different lengths of dsRNA (200, 400, and 600 bp) and stimulated wild-type and IPS-1–deficient MEFs with these RNA by transfection. IPS-1–deficient MEFs did not produce IFN-β in response to any length of dsRNA tested (Fig. 2 D). Collectively, these observations demonstrate that IPS-1 is required for RIG-I–mediated responses.

Because members of TLRs have been shown to recognize viral components and participate in type I IFN induction, we next examined responses of IPS-1–deficient mice to TLR ligands. We examined TLR9-mediated responses using splenocytes, which contain plasmacytoid DCs that produce type I IFN in response to the TLR9 ligand. IFN-β and IFN-α production after D35 stimulation was comparable between wild-type and IPS-1–deficient splenocytes (Fig. 2 E). Induction of Cxcl10 and Il6 mRNA after stimulation with LPS, a TLR4 ligand, was also comparable between wild-type and IPS-1–deficient MEFs (Fig. 2 F). Thus, IPS-1 is not involved in TLR-mediated type I IFN induction.

Role of IPS-1 in Mda5-dependent responses

We next examined Mda5-mediated responses using EMCV and poly I:C (9). Type I IFN after EMCV infection was abolished in PECs derived from IPS-1–deficient mice (Fig. 3 A). Although IL-6 induction was observed in the absence of IPS-1, the induction was reduced compared with wild-type cells (Fig. 3 A). Induction of genes encoding IFN-α, IFN-β, IP-10, RANTES, and IL-6 was also reduced in IPS-1–deficient PECs (Fig. 3 B). Type I IFN and inflammatory cytokines are induced in MEFs when poly I:C is administrated into the cytoplasm by lipofection. Notably, this induction is independent of TLR3 but dependent on Mda5 (9, 25). Production of IFN-β, IFN-α, and IL-6 after poly I:C transfection was markedly reduced in IPS-1–deficient MEFs in comparison to wild-type cells (Fig. 3 C). Similarly, Ifnb, Ifna, Cxcl10, and Il6 induction was...
severely impaired in IPS-1–deficient MEFs (Fig. 3 D). Impaired IFN-β and IL-6 induction upon poly I:C transfection also observed in IPS-1–deficient GM-DCs where poly I:C–mediated responses are also dependent on Mda5 but not TLR3 (Fig. S2, http://www.jem.org/cgi/content/full/20060792/DC1) (9). Similar to Mda5-deficient mice, induction of IFN-β and IL-6 was also impaired in IPS-1–deficient GM-DCs when poly I:C was given to the culture medium (unpublished data; reference 9). We next challenged the mice with poly I:C and measured cytokine production in sera. Whereas wild-type mice showed rapid induction of serum IFN-α, IFN-β, and IL-6, the induction was remarkably reduced in IPS-1–deficient mice (Fig. 3 E). Collectively, these results indicate that IPS-1 is essential for Mda5-mediated responses.

**Impaired activation of NF-κB and IRF-3 in response to NDV infection in IPS-1–deficient cells**

We next examined activation of signaling pathways after virus infection. IPS-1–deficient MEFs displayed severely impaired induction of NF-κB–DNA binding after NDV infection, whereas they showed comparable induction of the binding activity with wild-type cells when stimulated with LPS (Fig. 4 A). Nuclear translocation of RelA, a component of NF-κB, was also diminished in IPS-1–deficient cells (Fig. 4 B). Induction of an IRF-3 dimer formation after NDV infection was not observed in IPS-1–deficient MEFs (Fig. 4 C). Furthermore, tyrosine phosphorylation of STAT1, which is induced by secreted type I IFN, was abolished in IPS-1–deficient MEFs (Fig. 4 D). Up-regulation of IRF-7, which is also involved in type I IFN induction by cooperating with IRF-3, was also abrogated in IPS-1–deficient cells (Fig. 4 E). Collectively, IPS-1 is indispensable for NDV-induced activation of IRF-3 and NF-κB.

**IPS-1–deficient mice are susceptible to EMCV and VSV infection**

To gain insights into the biological relevance of IPS-1 in virus infection-mediated responses in vivo, we challenged
mice with EMCV to measure serum cytokines. Production of cytokines, including IFN-\(\beta\), IFN-\(\alpha\), IL-6, IP-10, and MCP-1, was severely impaired in IPS-1–deficient mice (Fig. 5 A). We next examined the susceptibility of IPS-1–deficient mice to EMCV infection. IPS-1–deficient mice were more susceptible to EMCV infection than control mice (\(P < 0.05\)) (Fig. 5 B). In accordance with the increased susceptibility to EMCV, virus titer in the heart of IPS-1–deficient mice was higher than control mice, which was similar to that observed in Mda5-deficient mice (9). Furthermore, we observed that IPS-1–deficient mice succumbed to VSV infection (Fig. 5 D), and virus titer in the liver and brain was markedly higher in IPS-1–deficient mice than wild-type mice (Fig. 5 E). Collectively, these observations indicate that IPS-1 is indispensable for humoral responses in vivo against EMCV and VSV infection.

Role of IPS-1 in B-DNA– and DNA virus–mediated responses
Because IPS-1 is implicated in B-DNA–mediated responses, we next examined the contribution of IPS-1 to this pathway (26, 27). Production of IFN-\(\beta\) after B-DNA transfection was slightly reduced (~40%) in IPS-1–deficient MEFs compared with wild-type cells (Fig. 6 A). However, no remarkable reduction of IP-10 and IL-6 was observed in IPS-1–deficient cells (Fig. 6 A). In Northern blot analysis, there was no reduction of Cxcl10, Il6, and Cdl5 expression in any time course examined in IPS-1–deficient MEFs, whereas the induction of Ifnb was slightly reduced after stimulation compared with wild-type MEFs (Fig. 6 A), suggesting that IPS-1 is partially involved in early phase induction of IFN-\(\beta\). We next examined DNA virus–mediated responses. To this end, we used a modified vaccinia virus, Ankara (MVA), lacking E3 ligase (DE3L) which encodes a poxvirus regulatory protein critical for inhibiting host type I IFN responses (26). IPS-1–deficient MEFs displayed reduced induction of IFN-\(\beta\) in response to MVA DE3L at 24 h compared with wild-type MEFs (Fig. 6 C). In contrast, IL-6 induction was comparable between wild-type and IPS-1–deficient MEFs (Fig. 6 C). These findings suggest that IPS-1 is not essential for MVA-mediated responses but is required for the maximal induction of IFN-\(\beta\). We next examined the amounts of viral protein from MVA DE3L–infected MEFs by immunoblot analysis of total cell lysates using anti-MVA antibody (Fig. 6 D). MVA protein was elevated at 24 h and decreased by 48 h after infection in wild-type MEFs (Fig. 6 D). In IPS-1–deficient MEFs, elevated levels of MVA protein were still observed at 48 h (Fig. 6 D), suggesting that IPS-1 is partially involved in the elimination of MVA.

DISCUSSION
In this study, we examined the physiological role of IPS-1 through the generation of IPS-1–deficient mice. We showed that IPS-1 is indispensable for responses against various single-stranded RNA viruses, including NDV, VSV, and SeV, all of which are recognized by RIG-I. The induction of type I IFN, IFN-stimulated genes, and inflammatory cytokines are abrogated in various cell types, including MEFs, PECs, and conventional DCs, and the up-regulation of CD86 is impaired in IPS-1–deficient conventional DCs. IPS-1–deficient mice also show severe defects in the responses to EMCV, which is recognized by Mda5. Furthermore, IPS-1 is essential to the induction of type I IFN in response to dsRNA and poly I:C that are recognized by RIG-I and Mda5, respectively. In agreement with the in vitro studies showing that overexpression of IPS-1 activates IFN-\(\beta\) and NF-\(\kappa B\) promoters, IPS-1–deficient cells fail to activate IRF-3 and NF-\(\kappa B\) in response to NDV. Thus, IPS-1 is the sole adaptor in both RIG-I– and Mda5–dependent signaling pathways.
In addition to RIG-I and Mda5, certain members of TLRs, including TLR3, TLR4, TLR7, and TLR9 also recognize viral components to induce type I IFN and inflammatory cytokines (1, 2). TLR7 and TLR9 recruit an adaptor MyD88, which subsequently forms a signaling complex with a protein kinase IRAK1 and IRF-7 that mediates induction of type I IFN (28–31). TLR3 and TLR4 induce type I IFN through TIR domain–containing adaptor-inducing IFN-β (TRIF), which interacts with TANK-binding kinase 1 and IRF-3 (32–34). Furthermore, a previous report demonstrated that IPS-1 interacts with TRIF (18), suggesting an involvement of IPS-1 in TLR-mediated signaling. In IPS-1–deficient cells, however, both TLR9– and TLR4–mediated induction of cytokines are normal, indicating that IPS-1 is not involved in TLR-mediated antiviral responses.

Induction of IL-6 after infection with EMCV is still observed in IPS-1–deficient PECs, although the induction is reduced when compared with that of wild-type cells. Similarly, there is a small induction of serum cytokines after poly I:C administration in IPS-1–deficient mice. Since poly I:C, viral nucleic acids, and viral glycoproteins can be recognized by members of TLRs, the residual induction of IL-6 might be mediated by the activation of TLR-mediated pathways. Generation of IPS-1/MyD88 and IPS-1/TRIF doubly deficient mice will be required to clarify the relationships between the RIG-I/Mda5– and TLR–mediated pathways in virus infection-mediated cytokine induction. Also, these mice will be useful to understand the specific or overlapping role of the IPS-1–, MyD88– and TRIF–dependent pathways in the induction of antiviral innate immune responses and subsequent induction of adaptive immune responses.

Notably, IPS-1 is not essential in B-DNA– or DNA virus–mediated responses. We have previously shown that knockdown of IPS-1 in HEK293 cells partially abrogated B-DNA–dependent IFN-β promoter activation (26, 27). However, IFN-β induction in response to B-DNA was still observed in IPS-1–deficient MEFs, although the induction was slightly decreased in the early phase (approximately 40% reduced). These aspects strongly suggest that cells possess a yet unidentified adaptor that is unique to dsDNA sensor(s). In response to MVA infection, IFN-β induction was reduced in IPS-1–deficient cells, and the elimination of viruses in IPS-1–deficient cells was consistently delayed compared with wild-type cells. However, dsRNA might be generated as a by-product of symmetrical transcription in DNA viruses. Thus, it is possible that reduced IFN-β in IPS-1–deficient cells is due to a lack of responses of these cells to dsRNA.

IPS-1 was shown to localize to mitochondria, suggesting a role of IPS-1 in the regulation of apoptosis, which is important for the host defense to virus infection (17).
However, cell death induction in response to VSV infection or poly I:C stimulation was comparable between wild-type and IPS-1–deficient MEFs (Fig. S3, available at http://www.jem.org/cgi/content/full/20060792/DC1). This finding indicates that virus-induced cell death does not require IPS-1. Rather, the localization of IPS-1 to the mitochondria is essential for type I IFN induction in virus-infected cells.

Finally, it is possible that the IPS-1–dependent pathway is central to antiviral responses against a wide variety of RNA viruses. Conversely, numerous viruses may have evasion mechanisms that counteract the IPS-1–dependent pathway. Indeed, HCV NS3/4A protease reportedly cleaves IPS-1 at C508, releasing IPS-1 from mitochondria that results in loss of IRF-3 activation and type I IFN induction (19, 35, 36). Therefore, development of drugs that control the IPS-1–dependent pathway would be therapeutically useful against infectious diseases caused by RNA viruses.

MATERIALS AND METHODS

Generation of IPS-1–deficient mice. The IPS-1 gene was isolated from genomic DNA extracted from embryonic stem cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 1.8-kb fragment encoding the IPS-1 open reading frame (exons 1 and 2) with a neomycin-resistance gene cassette (neo), and a herpes simplex virus thymidine kinase (HSV-TK) driven by the PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into ES cells, G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and further confirmed by Southern blot analysis. Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain IPS-1 /− mice. Mice were bred and maintained in a specific pathogen-free facility of Research Institute for Microbial Diseases in accordance with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care. Mouse protocols were approved by the Osaka University Animal Care and Use Committee. All mice were killed at 5–9 wk of age.

Cells, viruses, and reagents. MEFs were prepared from day 13.5 to 14.5 embryos. PECs from mice were collected 3 d after intraperitoneal injection with 4% thioglycollate medium. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 mM 2-ME, and 100 ng/ml human Flt3 ligand (PeproTech), or 10 ng/ml murine GM-CSF (PeproTech) for 6–8 d to use as FL-DCs and GM-DCs, respectively. B220−CD11c+ FL-DCs were isolated from FL-DCs by MACS using anti-B220 antibody microbeads from Miltenyi Biotech as described previously (26). NDV, VSV NCP, SeV Cm, EMCV, and MVA Ankara ΔE3L were provided by T. Abe and Y. Matsuura (Research Institute for Microbial Diseases), T. Fujita (Institute for Virus Research, Kyoto University, Kyoto, Japan), and H. Ludwig and G. Sutter (Paul-Ehrlich Institute, Langen, Germany) and described previously (8, 9, 26). LPS from Salmonella minnesota Re-595 was purchased from Sigma-Aldrich. Synthetic ds B-DNA (poly[dA-dT]·poly[dT-dA]) and poly I:C were purchased from GE Healthcare. A/D-type CpG DNA (I35) was described previously (31). In vitro–transcribed dsRNA was prepared from the mouse Lamin A/C cDNA sequence as described previously (9). For stimulation of cells, B-DNA or poly I:C was mixed with Eugene 6 transfection agent (Roche) at a ratio of 1:1 (vol/wt) in OptiMEM and incubated for 15 min before stimulation. Anti–extracellular signal-regulated kinase 1/2, anti-RelA, anti–IRF-3, and anti-STAT1 antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti–phospho-STAT1 antibody was purchased from Cell Signaling. Polyclonal rabbit anti-MVA antibody was purchased from Biogenesis. Anti–IPS-1 antibody was generated in rabbits by immunizing synthetic peptides that correspond to amino acids 425–438 of mouse IPS-1 as immunogens.

Figure 6. Role of IPS-1 in B-DNA–mediated responses. (A and B) Wild-type and IPS-1–deficient MEFs were transfected with 10 μg/ml B-DNA. After 24 h, the concentration of IFN-β, IP-10, and IL-6 was measured by ELISA (A). After 2, 4, and 6 h, total RNA was extracted and subjected to Northern blot analysis to determine the expression pattern of the respective genes (B). Asterisks indicate not detected. Indicated values are means ± SD of triplicates. (C and D) MEFs were infected with 10^7 PFU MVA ΔE3L. After 24 and 48 h incubation, the concentration of IFN-β and IL-6 was measured by ELISA (C). Whole cell lysates were subjected to immunoblot analysis using anti-MVA antibody (D).
ELISA. Culture supernatants of cells (10^6) seeded on 24-well plates or serum were collected and analyzed for cytokine levels with ELISA. ELISA kits for IFN-β and IFN-α were purchased from PBL Biomedical Laboratories. IL-6, IP-10, and MCP-1 ELISA kits were purchased from R&D Systems. ELISA was performed according to the manufacturer’s instructions.

**Northern blot analysis.** Total RNA was extracted using Trizol reagent (Invitrogen), electrophoresed, transferred onto nylon membranes, and then hybridized with the indicated cDNA probe. The full-length IPS-1 cDNA fragment was used as a probe to detect IPS-1 expression. Other probes were described previously (25).

RT-PCR. Total RNA was isolated with Trizol reagent (Invitrogen) and was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. PCR was then done with the following primers: IRF-7, 5′-TCGGTCTAGGGATCTCGG-3′ and 5′-GGCCCTGGAAGTGGGACAC-3′ and β-actin, 5′-GACATGGAGAA- GAPGTCGGCCAC-3′ and 5′-ATCCTCTGCAGAGATCTCAGAC-3′. Details of other primers were described previously (26).

**Immunoprecipitation, immunoblot, electrophoretic mobility shift assay, and Native-PAGE analyses.** Immunoprecipitation, immunoblot, electrophoretic mobility shift assay, and Native-PAGE analyses were performed as described previously (32, 34).

**Plaque assay.** 48 h after EMCV infection or 72 h after VSV infection, tissues were collected and analyzed for cytokine levels with ELISA. ELISA kits for IL-12, IP-10, and MCP-1 were purchased from R&D Systems. ELISA was performed according to the manufacturer’s instructions. The plaques were counted.

**Flow cytometric analysis of costimulatory molecule expressions.** Cell surface expression of CD86 was analyzed as described previously (37). In brief, B220^+^ FL-DCs were washed with ice cold PBS, fixed, and stained with FITC-, PE-, CyChrome- and APC-labeled antibodies in the presence of anti-CD16 for 30 min at room temperature. Stained cells were washed, resuspended in PBS/0.1% BSA/0.1% NaN₃, and analyzed by FACS Calibur followed by analysis using CELLQuest software (Becton Dickinson). All antibodies were obtained from Becton Dickinson.

**Online supplemental material.** Impaired induction of IFN-β and IL-6 in response to NDV, VSV, or SeV infection in PECs and GM-DCs derived from IPS-1–deficient mice is shown in Fig. S1. Impaired induction of IFN-β and IL-6 in response to poly I:C stimulation in GM-DCs derived from IPS-1–deficient mice is shown in Fig. S2. Normal induction of cell death after VSV infection or poly I:C stimulation in IPS-1–deficient MEFs is shown in Fig. S3. Online supplemental material is available at http://www.jem.org/cgi/content/full/20060792/DC1.

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