DNA Sensitive-Independent Inhibition of Herpes Simplex Virus 1 Replication by DAI/ZBP1

Thanh H. Pham,* Ki Mun Kwon, Young-Eui Kim, Kyeong Kyu Kim, Jin-Hyun Ahn

Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, Republic of Korea

DNA-dependent activator of interferon regulatory factor (DAI), which is also referred to as Z-DNA binding protein 1 (ZBP1) or DLM-1, was initially identified as a highly upregulated protein in mouse tumor stromal cells and in macrophages treated by gamma IFN (IFN-γ) or lipopolysaccharide (1). Structural analyses have revealed that DAI/ZBP1/DLM-1 (referred to as DAI hereafter) contains the amino-terminal Z-form DNA-binding domains, Zx and Zβ, which are homologous to those of adenosine deaminase that acts on RNA (ADAR1), an RNA editing enzyme (2–5). Since Z-DNA is located near the transcription start sites of certain genes in the genome, a role of DAI in transcriptional regulation has been suggested (6, 7). Induction of DAI was also observed in mouse hepatocytes infected with hepatitis B virus (HBV) (8) and in mouse embryonic fibroblasts (MEFs) stimulated by B-form DNA (9).

Recently, DAI was shown to act as a cytosolic B-form DNA sensor that initiates IFN responses via activation of the nuclear factor-κB (NF-κB) and interferon regulatory transcription factor 3 (IRF3) pathways in mice (10). In addition to the Z-DNA-binding domains, a region termed the D3 domain was demonstrated to primarily contribute to the recognition of B-DNA in vitro (10). However, all of the Zx, Zβ, and D3 domains were required for efficient B-DNA binding in vivo and DAI was suggested to undergo DNA-mediated multimerization to evoke activation of IFN responses (11). The carboxyl-terminal region of DAI was responsible for recruitment of both IRF3 and TANK-binding kinase 1 (TBK1), an IκB kinase that activates IRF3 (10). The mechanism by which DAI activates the NF-κB pathway was shown to involve recruitment of receptor-interacting protein kinase 1 (RIP1) and RIP3 through a RIP homotypic interaction motif (RHIM)–dependent interaction with DAI (12, 13). Recently, the binding of DAI with RIP3 was shown to mediate virus-induced programmed necrosis (14).

The requirement of DAI in induction of IFN response by cytosolic stimulation of B-DNA is dependent on cell type. DAI played a role in the DNA-mediated IFN production in mouse fibroblast L929 (10, 12, 15) and mouse SVEC4-10 endothelial cells (12), whereas it was not required for MEFs (11, 16) and mouse bone marrow dendritic cells generated by granulocyte macrophage colony-stimulating factor or Fms-like tyrosine kinase 3 (16). In L929 cells, mouse microglial cells, and astrocytes, IFN production upon herpes simplex virus 1 (HSV-1) infection also required DAI expression (17). Among human cells, A549 lung carcinoma cells did not require DAI for the DNA-mediated IFN production, whereas HEK293 embryonic kidney cells only partially did so (15). Human fibroblast cells required DAI for the IFN production after human cytomegalovirus infection (18, 19). These reports suggest that the cytosolic DNA sensing system for the induction of IFN responses may be redundant, depending on different receptors in different cell types.

Although DAI has been shown to reduce the growth of HSV-1 in certain cell types, the role of DAI in cell types that do not necessarily require DAI for DNA sensing has not been demonstrated. In the present study, we show that DAI can inhibit HSV-1 replication through mechanisms independent of DNA sensing. Our data demonstrate that DAI acts as a restriction factor that sup-
presses viral gene activation especially at a low multiplicity of infection (MOI). Furthermore, we demonstrate that DAI interacts with ICP0 and that this interaction may also be involved in the anti-HSV-1 activity of DAI.

**MATERIALS AND METHODS**

**Cell culture and virus.** HepG2, MEF, Vero, BHK, U2OS, and 293T cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO₂ humidified incubator at 37°C. To induce DAI expression HepG2 cells were treated with IFN-γ (100 ng/ml; Sigma-Aldrich) for 20 h. HSV-1 (strain KOS/Δk12) contained a lacZ reporter gene under the control of the ICP4 promoter inserted into the thymidine kinase (TK) gene (20). The mutant virus ΔIX3.1 has an ICP0 deletion in the KOS background (21). The wild-type virus (KOS/Δk12) was grown and titrated in Vero cells by plaque assays. The ΔIX3.1 virus was grown in BHK cells and titrated in U2OS cells, in which ICP0 is not required for efficient replication of HSV-1 (22).

**Plasmids.** Plasmids expressing 5’ hemagglutinin (HA)-tagged human DAI (1 to 429 amino acids), its amino-terminal fragment (Za/ΔZp), or an amino-terminal truncated version (ΔI-80) were previously described (23). The ΔE2on2 (ΔE2) mutant with a deletion between amino acids 12 and 86 and the ΔE4on5/ΔE4/5 mutant with a deletion between amino acids 110 and 223 were generated by PCR in the same pSG5 (24) background. Retroviral vectors, MIN-DAI and MIN-ΔE4/5, were constructed by inserting the corresponding DAI cDNAs into pMIN (25). The cDNA for human RIP1 was provided by Jaehong Song (Yonsei University, Seoul, Republic of Korea). Plasmid for 5’-HA-tagged RIP1 was produced in pSG5 background using Gateway technology (Invitrogen). Plasmid encoding ΔRHM mutant RIP1 was generated by PCR in the same background. Plasmid expressing ICP0 was previously described (26) and an ICP0-luciferase reporter gene plasmid was provided by Gary S. Hayward (Johns Hopkins University School of Medicine).

**Transfection.** HepG2 and MEF cells were transfected with plasmid DNAs using the Metafectene reagents (Biotex). For cytosolic B-DNA stimulation, HepG2 cells were transfected with poly(dA-dT)-poly(dA-dT) (Sigma-Aldrich) using Lipofectamine 2000 reagents (Invitrogen). Stimulation, HepG2 cells were transfected with poly(dA-dT)-poly(dA-dT) (Sigma-Aldrich) using Lipofectamine 2000 reagents (Invitrogen). For cytosolic B-DNA stimulation, HepG2 cells were transfected with poly(dA-dT)-poly(dA-dT) (Sigma-Aldrich) using Lipofectamine 2000 reagents (Invitrogen).

**Indirect immunofluorescence assay (IFA).** Cells were harvested at 48 h after transfection. The cells were incubated with appropriate primary antibody in PBS at 37°C for 1 h and then with secondary antibody in PBS at 37°C for 45 min. To stain nuclei, mounting solution containing DAPI (4’,6-diamidino-2-phenylindole; Vector Laboratories) or Hoechst strain (Invitrogen) and antifade reagent (Molecular Probes) were used. Slides were examined and photographed with a Carl Zeiss Axioshot microscope or with a Carl Zeiss Axioplan 2 confocal microscope system with LSM510 software.

**Immunoblot analysis.** Cells were collected and lysed with an ice-cold RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodeyl sulfate [SDS]) supplemented with protease inhibitor cocktail (Santa Cruz). Clarified cell extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and the resolved proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). All subsequent procedures were performed as previously described (23).

**β-Galactosidase reporter assay.** Cells were collected and lysed by three freeze-thaw steps in 100 μl of 0.25 M Tris-HCl (pH 7.9) plus 1 mM dithiothreitol. Cells extracts were clarified by microcentrifuge. Each reaction mix was prepared in 96-well dishes containing 20 μl of extracts plus 100 μl of Z-buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 M MgSO₄, 0.05 M β-mercaptoethanol) plus 20 μl of ONPG (o-nitrophenyl-β-D-galactopyranoside [Sigma-Aldrich]; 4 mg/ml in sterile water). Reactions were incubated at 37°C until the yellow color was developed. Reaction was stopped by adding 50 μl of 1 M Na₂CO₃. The β-galactosidase absorbance of each reaction was read at 420 nm.

**Cell viability assay.** Cells were seeded in 96-well flat-bottom plates and infected with virus at an MOI of 3. At 24 h after infection, 10 μl of WST-1 solution (EZ-Cytox; Daeil Lab Service) was added to the 96-well plate. After 1 h, the absorbance of each reaction was read at 410 nm.

**Reverese transcription-PCR (RT-PCR) and quantitative real-time PCR.** Total RNA was isolated from 2 × 10⁵ cells using TRizol reagent (Invitrogen). cDNAs were synthesized using the random hexamer primers in the SuperScript III system (Invitrogen). Quantitative real-time PCR was performed using SYBR Green PCR Core Reagents (Applied Biosystems) and ABI Prism 7700 software. The following primers were used: human DAI, forward (5’-CAAGAAGCGGGGAAAGACAT-3’) and reverse (5’-ATCACTCAATTGTGTGCTCC-3’); IC4P, forward (5’-CTCA TGTGTTCAACCCGGT-3’) and reverse (5’-CTTCCAGTGCCACAC-3’); ICP27, forward (5’-GCATGTCATCTCCTCC-3’) and reverse (5’-TCCTGTTGTCCTCCTC-3’); IFN-β, forward (5’-GC TCTCTGTTGTTGCTTCCAGC-3’) and reverse (5’-CAATTGTCTCTCTGATT-3’) and (5’-CACGGTACATGTGTGCTGCG-3’).

**Plaque assays.** The serially diluted virus samples were used to inoculate Vero or U2OS cells in a six-well plate (10⁶ per well). After 1 h incubation, the medium was removed, and 2 ml of methylcellulose overlay was added to each well. After incubation for 3 days, the methylcellulose was removed, and the cell monolayer was stained with 1% crystal violet in 50% ethanol for 20 min. The cells were washed with running tap water and dried. The numbers of plaque produced were counted.

**Antibodies.** Anti-DAI polyclonal antibody (PAb) (23) was previously described. Anti-HA rat monoclonal antibody (MAB) 3F10 conjugated with peroxidase or labeled with fluorescein isothiocyanate (FITC) and anti-Myc mouse MAB was purchased from Roche. Mouse MABs for UL42 and IFI16 and rabbit PAb for IRF3 were purchased from Santa Cruz Biotechnology. Mouse MAB for ICP0 was purchased from Viraxis. The rabbit PAb against ICP0 and PML were a gift from Gary S. Hayward (Johns Hopkins University School of Medicine). Rabbit MAB for phosphorylated IRF3 (S396) was obtained from Cell Signaling Technology. Mouse MABs for IκBα and β-actin were purchased from Young In Frontier (Seoul, Republic of Korea) and Sigma-Aldrich, respectively.

The wild-type virus (KOS/Δk12) was grown in Vero cells by plaque assays. The ΔIX3.1 virus was grown in BHK cells and titrated in U2OS cells, in which ICP0 is not required for efficient replication of HSV-1 (22).
Luciferase reporter assay. Cells were collected and lysed by three freeze-thaw steps in 200 µl of 0.25 M Tris-HCl (pH 7.9) plus 1 mM dithiothreitol. Cell extracts were clarified in a microcentrifuge, and 30-µl portions of the of extracts were incubated with 350 µl of reaction buffer A (25 mM glycyl-glycine [pH 7.8], 15 mM ATP, 4 mM EGTA) and then mixed with 100 µl of 0.25 mM luciferin (Sigma-Aldrich) in reaction buffer A. A TD-20/20 luminometer (Turner Designs) was used for a 10-s assay of the photons produced (measured in relative light units).

CoIP assays. Cell lysates were prepared by sonication in 1 ml of coinmunoprecipitation (CoIP) buffer (50 mM Tris-Cl [pH 7.4], 50 mM NaF, 5 mM sodium phosphate, and 0.1% Triton X-100 containing protease inhibitors; Sigma-Aldrich) with a Vibra cell microtip probe (Sonics and Materials) for 10 s (pulse on, 1 s; pulse off, 3 s). Cell lysates were incubated for 16 h with the appropriate antibody at 4°C. A total of 30 µl of a 50% slurry of protein G-Sepharose (Amersham) was added and then absorbed for 1 h at 4°C. The mixture was pelleted and washed several times with CoIP buffer. The beads were resuspended and boiled for 5 min in loading buffer. Each sample was analyzed by SDS-PAGE and immunoblotting with the appropriate antibody.

Statistical analysis. Statistical significance between samples was determined using the Student t test. P values of <0.05 (*), <0.01 (**), and <0.001 (***)) are indicated in the figures.

RESULTS
Depletion of DAI enhances HSV-1 replication. DAI has been shown to be induced in hepatic cells by interferon treatment and HBV infection (8, 23). In RT-PCR analysis, we found that DAI transcription was induced in HepG2 cells after IFN-γ treatment or infected with HSV-1 at an MOI of 3 for indicated times. The levels of DAI and β-actin mRNAs were determined by RT-PCR. (B) (Top panel) The DAI mRNA levels in normal HepG2, control RNA (shC) or DAI shRNA (shDAI-1 and shDAI-2)-expressing HepG2 cells were determined by RT-PCR. The level in HA-DAI-transfected cells is shown as a size control. (Bottom panel) HepG2 or HepG2-shRNA cells were mock infected or infected with HSV-1 at an MOI of 3, and the DAI levels were determined by RT-PCR. The levels of β-actin were shown as loading controls. (C and D) HepG2 or HepG2-shRNA cells in 12-well plates were mock infected (M) or infected with HSV-1 at an MOI of 1 or 3. At 24 h, the viral supernatants were harvested, and the production of progeny virions was measured by plaque assays using Vero cells. The mean values of three independent assays with the standard errors are shown. (E and F) Cells in six-well dishes were mock infected or infected with HSV-1 at an MOI of 1 at 24 h, total cell extracts were prepared and immunoblotting was performed with antibodies for ICP0 and UL42. The blots were also probed for β-actin as a loading control. (G) Cells were infected with HSV-1 at an MOI of 3, and immunoblotting for ICP0 was performed with cell extracts prepared at 3 and 24 h after infection.
knockdown was observed at 3 h after infection (Fig. 1G). Together, these results demonstrate that DAI knockdown in HepG2 cells facilitates HSV-1 replication by promoting viral gene expression and that this effect is more profound at a lower MOI.

**DAI expression represses HSV-1 growth and ICP0 promoter activation.** We next investigated the effect of ectopic expression of DAI on HSV-1 replication. Retroviral vectors expressing wild-type or mutant DAI (ΔE2, Zα domain deleted; ΔE4/5, Zβ and D3 domains deleted; and Zα+Zβ, containing only Zα and Zβ domains) were constructed (Fig. 2A). HepG2 cells expressing these DAI proteins were produced by retroviral transduction. These cells were infected with HSV-1 for 24 h and the production of β-galactosidase, which is expressed under the control of the ICP4 promoter in the viral genome, in infected cells was measured. Expression of wild-type and ΔE2 mutant DAI efficiently repressed the β-galactosidase production, whereas ΔE4/5 and Zα+Zβ mutants almost completely lost the repressive activity (Fig. 2B).

When the levels of progeny virion production were compared between wild-type and mutant DAI-expressing cells, wild-type DAI significantly reduced virus titer, whereas ΔE4/5 mutant had a minimal effect (Fig. 2C). The repressive effect of wild-type DAI on viral replication correlated with the reduced expression of ICP0 and UL42 (Fig. 2C). Both wild-type and ΔE4/5 mutant DAI expressed by retroviral vectors were mainly distributed in the cytoplasm as a mixture of diffuse and punctate forms, with some also being localized in the nucleus as foci (Fig. 2D). When HepG2 cells

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**FIG 2** Ectopic expression of DAI represses HSV-1 replication. (A) Structures of wild-type or mutant DAI proteins expressed in retroviral MIN vectors. The coding regions corresponding to each exon of DAI are shown at the top. The regions corresponding to the Zα, Zβ, and D3 domains are indicated by gray boxes. (B) HepG2 cells transduced by control (MIN) or DAI (wild type [Wt] or mutant)-expressing retrovirus were mock infected or infected with HSV-1 at an MOI of 3. At 24 h, the cell lysates were prepared and assayed for β-galactosidase production by ONPG assays. The expression levels of wild-type and mutant DAI were determined by immunoblotting with anti-DAI antibody. (C and D) Control (MIN) or DAI-expressing (MIN-Wt or MIN-ΔE4/5 mutant) cells were mock infected or infected with HSV-1 at an MOI of 3 for 24 h. (C) Virus titers in culture supernatants were measured by plaque assays, and the levels of viral ICP0 and UL42 proteins and DAI proteins in cell lysates were determined by immunoblotting. (D) Cells were fixed with the methanol procedure and stained with anti-DAI antibody and fluorescence isothiocyanate (FITC)-labeled donkey anti-rabbit IgG. A mounting solution containing DAPI was used to stain cell nuclei. (E) Normal HepG2 cells were transfected with empty plasmid or increasing amounts of plasmid expressing HA-tagged wild-type or ΔE4/5 mutant DAI. At 24 h, these cells were mock infected or infected with HSV-1 at an MOI of 3. After further incubation for 24 h, the β-galactosidase levels were measured. The levels of DAI and β-actin proteins were determined by immunoblotting. (F) Control or DAI-expressing cells were mock infected or infected with HSV-1 at an MOI of 3 for 24 h. The levels of ICP0 and ICP27 mRNA were measured by RT-PCR. The levels of β-actin are shown as loading controls.
was transiently transfected with wild-type and ΔE4/5 mutant DAI and then infected with HSV-1 for 24 h. wild-type DAI inhibited the production of β-galactosidase in a dose-dependent manner, whereas ΔE4/5 mutant did not (Fig. 2E). The levels of ICP0 and ICP27 mRNAs were reduced by the expression of wild-type DAI but not ΔE4/5 mutant, suggesting that the anti-HSV-1 effect of DAI expression includes transcriptional control (Fig. 2F). Collectively, these results demonstrate that DAI inhibits HSV-1 growth and that the anti-HSV-1 activity requires the central region encompassing Z and D3 domains but not the amino-terminal region containing ZA domain.

FIG 3 Inhibition of the ICP0 promoter by DAI. (A) HepG2 or HepG2-shRNA cells in six-well plates were transfected with 0.5 μg of plasmid containing the ICP0-luciferase reporter gene. At 48 h, total cell extracts were prepared and assayed for luciferase activity. Shown are mean values with the standard errors of luciferase activity of three independent assays. (B) HepG2 cells were cotransfected with 0.5 μg of the reporter plasmid and increasing amounts (0.1, 0.5, 1, and 2 μg) of plasmid expressing HA-DAI or empty vector (pSG5). At 48 h, the luciferase reporter assays were performed. The expression levels of HA-DAI and β-actin were determined by immunoblotting with anti-HA and anti-β-actin antibodies. (C) Cells were cotransfected with 0.5 μg of the reporter plasmid and 2 μg of plasmid expressing wild-type (Wt) or mutant DAI (Δ1-80, ΔE2, or ΔE4/5). At 48 h, the luciferase reporter assays were performed as in panel A. (D) HepG2 cells transduced with control (MIN) or DAI (Wt or mutant DAI (Δ1-80, ΔE2, or ΔE4/5)-expressing retrovirus were transfected with 0.5 μg of plasmid containing the HTLV LTR-luciferase reporter gene and increasing amounts (0.1 and 0.5 μg) of plasmid expressing HA-DAI. At 48 h, the luciferase reporter assays were performed as in panel A.

FIG 4 DAI depletion does not affect IFN-β transcription and IRF3 phosphorylation in B-DNA-stimulated HepG2 cells. (A) HepG2 or HepG2-shRNA cells were seeded into six-well plates and then untreated or treated with increasing amounts (0.1, 0.5, 1, or 3 μg) of poly(dA-dT) for 12 or 24 h. The total mRNAs were isolated with TRIzol reagents (Invitrogen), and the mRNA levels of IFN-β were measured by quantitative real-time RT-PCR. The IFN-β mRNA levels over those of control β-actin are indicated as the fold induction. (B) HepG2 or HepG2-shRNA cells were untreated or treated with 3 μg of poly(dA-dT) for 12 h. The mRNA levels of IFN-β (measured by quantitative real-time RT-PCR) over those of control β-actin are indicated as the fold induction. (C) Cells were treated with poly(dA-dT) as in panel B. Cell lysates were prepared by boiling cells in loading buffer, and the levels of IRF3 (total IRF3 and phosphorylated IRF3 on serine 386) and IκBα were determined by immunoblotting. The blot was also probed for β-actin as a loading control.
To investigate whether DAI expression affects HSV-1 gene expression, transient reporter gene assays were performed in HepG2 cells using the ICP0-luciferase reporter gene plasmid. When control and DAI-knockdown cells were transfected with the reporter gene plasmid, the ICP0 promoter activity was higher in DAI-knockdown cells than control cells (Fig. 3A). When cells were cotransfected with the reporter gene plasmid and DAI-expressing plasmid, DAI repressed the ICP0 promoter in a dose-dependent manner (Fig. 3B). Consistent with the data obtained with virus infection (in Fig. 2), DAI mutants without the Z/ZBP1 domain (∆1-80 and ∆E2) still showed wild-type activity, but ∆E4/5 mutant lost this repressive activity (Fig. 3C). When cells expressing wild-type DAI or ∆E4/5 mutant were transfected with the reporter gene plasmid, the ICP0 promoter was repressed by wild-type DAI, but not by ∆E4/5 mutant (Fig. 3D). In a control experiment, DAI repressed the human T cell leukemia virus (HTLV) long terminal repeat (LTR) but not the cyclin E promoter (Fig. 3E). This result suggests that DAI may be a general repressor, although the activity is found in a promoter-dependent manner. Therefore, these results of reporter gene analysis demonstrate that the anti-HSV-1 activity of DAI at least in part involves inefficient expression of viral ICP0 gene.

DAI is not essential for IFN-β transcription through cytosolic B-DNA sensing in HepG2 cells. We investigated whether DAI plays an essential role in the type I IFN pathway in HepG2 cells after treatment of immunostimulatory B-DNA. First, we tested
induction of IFN-β mRNA synthesis in HepG2 cells after cytosolic B-DNA stimulation. The results of quantitative real-time RT-PCR showed that the IFN-β mRNA level was increased by poly(dA-dT) stimulation in a dose-dependent manner; the maximum increase in this experiment was observed with stimulation of cells with 3 µg of poly(dA-dT) for 12 h (Fig. 4A). We found that the B-DNA-mediated induction of IFN-β transcription in this condition was similar between control and DAI-knockdown cells (Fig. 4B). Since IFN-β transcription is activated by IRF3 and NF-κB, we further examined the effect of DAI-knockdown on activation of IRF3 and NF-κB. Consistent with the data for IFN-β mRNA levels, both phosphorylation of IRF3 at serine 396 and degradation of IκBα after poly(dA-dT) stimulation were comparable between control and DAI-knockdown HepG2 cells (Fig. 4C). Taken together, these results suggest that DAI is not essential for the IFN-β production through cytosolic B-DNA sensing in HepG2 cells.

To further investigate the effect of DAI expression on NF-κB activation in HepG2 cells, we examined the effect of DAI-RIP1 interaction on the plasmid DNA transfection-dependent DAI-mediated activation of NF-κB (12, 13). As observed in these previous reports, human DAI interacted with wild-type RIP1 but not with ΔRHIM mutant in cotransfected 293T and HepG2 cells (Fig. 5A and B). Consistently, RIP1 was relocalized to cytoplasmic DAI foci in cotransfected cells in a RHIM-dependent manner (Fig. 5C). In reporter assays, a synergistic effect of DAI and RIP1 expression on NF-κB activation was observed in 293T cells (Fig. 5D), and this was also consistent with previous reports (12, 13). However, cotransfection of DAI and RIP1 did not enhance NF-κB activation in HepG2 cells (Fig. 5E). Furthermore, the B-DNA-mediated activation of NF-κB was normally observed in HepG2 cells, but this was not augmented by the overexpression of DAI and RIP1 (Fig. 5F). These results indicated that DAI interacted with RIP1, but their overexpression did not enhance the NF-κB activation by B-DNA treatment in HepG2 cells. Collectively, our analysis demonstrates that DAI is not essential for the IFN-β production through cytosolic B-DNAs in HepG2 cells.

Interaction of DAI with ICP0. We examined the localization pattern of DAI in HSV-1-infected HepG2. In normal HepG2 cells, the endogenous DAI proteins were detected at very low levels throughout the cells (Fig. 6Aa to c), although a few cytoplasmic DAI foci were detected in only a minority of cells (data not shown). After HSV-1 infection, the number of cells showing the DAI signals in the cytoplasm and often in the nucleus was increased at 3 h (Fig. 6Ad to i). ICP0 localizes to the nucleus at the early phase of infection and then is also distributed in the cytoplasm as diffuse and punctate forms at the late phase (30–33). We found that some DAI induced in HSV-1-infected cells colocalized with ICP0 in the nucleus and cytoplasmic foci (Fig. 6Ad to j, note the closed barbed arrowheads for nuclear colocalization and open arrowheads for cytoplasmic colocalization in panel j).

We previously demonstrated that transfected DAI was localized largely in the cytoplasm, but also in the nucleus in several cell types, including HepG2 (23). When HA-DAI-transfected HepG2 cells were infected with HSV-1 for 8 h, we found that HA-DAI effectively colocalized with ICP0 in the nucleus and the cytoplasm (Fig. 6B). Notably, the cytoplasmic ICP0 foci was less efficiently found in HA-DAI-expressing cells, suggesting that overexpression of DAI by transfection may delay the relocalization of ICP0 into the cytoplasm (Fig. 6Ba to c). Collectively, the results of the IFA suggest a specific association of DAI with ICP0 during HSV-1 infection.

Therefore, we further investigated the interaction of DAI with ICP0 using CoIP assays. When 293T cells were cotransfected with

![FIG 6 Colocalization of DAI with ICP0 during HSV-1 infection.](http://jvi.asm.org/)

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myc-DAI and ICP0, immunoprecipitation of myc-DAI coprecipitated ICP0 and, in reciprocal assays, immunoprecipitation of ICP0 coprecipitated myc-DAI (Fig. 7A). DAI ΔE4/5 mutant did not interact with ICP0, as well as wild-type DAI, indicating that the central region encompassing ZB and D3 domains is required for efficient ICP0 binding (Fig. 7B). We also examined the DAI-ICP0 interaction in HSV-1-infected cells. Since the DAI antibody used could not detect endogenous low-level DAI in immunoblot analysis, we could not detect the association of endogenous DAI with ICP0 in HSV-1-infected HepG2 cells. However, when HepG2 cells transiently transfected with HA-DAI were infected with HSV-1, ICP0 was coprecipitated with DAI (Fig. 7C). The association of DAI with ICP0 during virus infection was further assessed using wild-type or ΔE4/5 mutant DAI-expressing HepG2 cells. When these control (MIN) and DAI-expressing (MIN-DAI or MIN-DAI-ΔE4/5) cells were infected with HSV-1, immunoprecipitation of wild-type DAI, but not ΔE4/5 mutant, coprecipitated ICP0 (Fig. 7D). These results confirm that DAI indeed interacts with ICP0.

ICP0 is involved in DAI-mediated repression of HSV-1 growth. We demonstrated that DAI expression more significantly inhibits ICP0 expression at lower MOI (Fig. 1). In addition to the effect of DAI on viral IE gene expression, the interaction of DAI with ICP0 may also affect viral growth. To test it, we compared the effects of DAI knockdown and overexpression on the growth of wild-type and ICP0-deleted mutant viruses. The dlX3.1 virus contained a 3.1-kb deletion in both copies of the ICP0 gene in the HSV-1 genome and, therefore, completely loses all ICP0 functions (21). We first infected normal and DAI-knockdown HepG2 cells at an MOI of 3 and then compared the growth of wild-type and dlX3.1 mutant viruses by measuring the level of progeny virion production. In normal and control (shC) HepG2 cells, the dlX3.1 virus growth was reduced by >10-fold compared to the wild-type virus growth (Fig. 8A). Importantly, we found that, unlike wild-type virus, the growth of dlX3.1 virus was not affected by DAI knockdown (Fig. 8A). The growth of wild-type and dlX3.1 mutant viruses was also compared between control (MIN) and DAI-expressing [MIN-DAI and MIN-DAI(ΔE4/5)] cells. The results showed that, unlike wild-type virus, the growth of dlX3.1 virus was not repressed by wild-type DAI expression (Fig. 8B).

Since the high-level expression of DAI mediates virus-induced programmed necrosis in certain cell types, in which RIP3 was also expressed at high level (14), we investigated whether the DAI-mediated inhibition of HSV-1 growth is related to increased cell death. However, the levels of cell viability after infection with HSV-1 were similar among control and DAI-expressing cells, indicating that the anti-HSV-1 activity of DAI in HepG2 cells may be independent of virus-induced necrosis (Fig. 8C).

DAI is not required for the DNA-mediated production of IFN-β in MEFs (11, 16). Therefore, we also tested whether the expression of DAI inhibits HSV-1 replication in MEFs in an ICP0-dependent manner. MEFs expressing wild-type or ΔE4/5 mutant DAI were produced by retroviral vectors and infected with wild-type or ICP0 mutant virus. Like in HepG2 cells, the expression of wild-type DAI, but not ΔE4/5 mutant, repressed HSV-1 replication in MEFs (11, 16). Therefore, we also tested whether the expression of DAI inhibits HSV-1 replication in MEFs in an ICP0-dependent manner. MEFs expressing wild-type or ICP0 mutant virus. Like in HepG2 cells, the expression of wild-type DAI, but not ΔE4/5 mutant, repressed HSV-1 replication in MEFs, and that this repressive effect of DAI was not observed in dlX3.1 virus infection (Fig. 8D). Collectively, the results with the ICP0-deleted mutant virus demonstrated that the repression of HSV-1 growth by DAI in HepG2 cells and MEFs requires the presence of ICP0 protein, suggesting that the DAI-ICP0 interaction may contribute to anti-HSV-1 activity of DAI.

We further investigated whether DAI expression affects the ICP0’s Ub E3 ligase activity. It was recently shown that another DNA sensor IFI16 is degraded by ICP0 during HSV-1 infection in human fibroblasts (34). We also observed that IFI16 was degraded in HepG2 cells after infection with wild-type HSV-1 but not with dlX3.1 mutant virus (Fig. 9A). When compared between control and DAI-knockdown cells, the loss of IFI16 and PML (another
substrate of ICP0) during virus infection was increased by DAI knockdown (Fig. 9B). These results indicate that DAI expression downregulates the ICP0’s Ub E3 ligase activity, suggesting that the DAI-ICP0 interaction may affect the ICP0 function.

DISCUSSION

The present study demonstrates that DAI is not required for DNA sensing-mediated IFN-β production in HepG2 cells. Cytosolic stimulation of normal cells with B-form DNA induced IFN-β transcription, but this was not affected by DAI knockdown. In other cell types where DAI acts as a DNA sensor, DNA sensing through DAI activates both IRF3 and NF-κB pathways (10,12,13). However, in HepG2 cells, DAI knockdown did not affect B-DNA-mediated IRF3 activation, and the overexpression of DAI proteins were determined by immunoblotting. The levels of β-actin are shown as a loading control. In agreement with this notion, several other cytosolic DNA-sensing proteins, including absent in melanoma 2 (AIM2), RNA polymerase III, DHX9 and DHX36, and IFN-inducible IFI16 protein, have been discovered (35,36). Therefore, it is likely that the involvement of a specific DNA sensor depends on cell types. Whether these receptors coordinately operate in a specific cell type is not clear.

Despite no apparent effect of DAI on the DNA-mediated IFN production in HepG2 cells, our data demonstrate that DAI expression inhibits HSV-1 growth. The anti-HSV-1 activity of DAI independent of DNA sensing was also evidenced by the finding that DAI activity did not require the amino-terminal Zα domain, which is necessary for efficient B-DNA binding in vivo (11). Our data demonstrate that one apparent mechanism by which DAI inhibits HSV-1 growth is through suppression of viral IE gene expression. DAI knockdown increased the accumulation of ICP0 (and ICP4 [data not shown]) in virus-infected cells, whereas DAI overexpression reduced it. Furthermore, our reporter gene analysis...
that the virus-induced necrosis requires high-level expression of both RIP3 and DAI (14). However, we observed that RIP3 in HepG2 cells was expressed at a low level, as in 293T (data not shown), where necrotic response was not observed (39).

The results in the present study further demonstrate that DAI may inhibit HSV-1 growth by regulating ICP0 functions. This conclusion is supported by several observations. First, DAI interacted with ICP0. Second, DAI knockdown enhanced replication of wild-type virus but not the ICP0 deletion mutant. Third, DAI overexpression inhibited replication of wild-type virus but not ICP0 deletion mutant, and a mutant DAI defective in ICP0 binding lost its anti-HSV-1 activity. Moreover, it should be emphasized that the ICP0-dependent anti-HSV-1 activity of DAI was also observed in MEFs in which DAI is not required for DNA-mediated IFN production. Therefore, even in cell types where DAI is not necessarily required for DNA sensing, DAI appears to inhibit HSV-1 growth via different mechanisms, suppressing viral genomes and targeting to ICP0. In this respect, it is also notable that IFI16 was recently shown to act as an intrinsic restriction factor that inhibits the transcriptional activation of viral replication genes (40).

The ubiquitin E3 ligase function of ICP0 (41, 42) is thought to be involved in antagonizing cellular antiviral activities. The ICP0-mediated degradation of PML in the nucleus promotes viral growth by relieving the suppressive effect of PML on incoming viral genomes (43). ICP0 has also been shown to downregulate the production of IFN and IFN-stimulated genes during virus infection (44, 45). This repression by ICP0 at least in part involved the inhibition of IRF3 activity (33, 46, 47). A recent study also demonstrated that ICP0 degrades IFI16, and this activity is involved in inhibition of IRF3 activation (34). Our data here demonstrate that DAI expression inhibits the degradation of PML and IFI16 by ICP0. These results suggest that the association of DAI with ICP0 may regulate the E3 ligase activity of ICP0 that is crucial for HSV-1 replication. Further studies are required to address the exact roles of DAI-ICP0 interaction in the DAI-mediated inhibition of HSV-1 growth.

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