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Human Bocavirus NP1 Inhibits IFN-β Production by Blocking Association of IFN Regulatory Factor 3 with IFNB Promoter

Zhenfeng Zhang,* Zhenhua Zheng,* Huanle Luo,* Jin Meng,* Hongxia Li,* Qian Li,* Xiaowei Zhang,* Xianliang Ke,* Bingke Bai,† Panyong Mao,† Qinxue Hu,* and Hanzhong Wang*

Human bocavirus (HBoV) mainly infects young children. Although many infected children suffer from respiratory or gastroenteric tract diseases, an association between HBoV and these diseases is not definite. Because modulation of type I IFN is crucial for viruses to establish efficient replication, in this study, we tested whether HBoV modulates type I IFN production. We observed that a nearly full-length HBoV clone significantly reduced both Sendai virus (SeV)- and poly(deoxyadenylic-thymidylic) acid-induced IFN-β production. Further study showed that NP1 blocked IFN-β activation in response to SeV, poly(deoxyadenylic-thymidylic) acid, and IFN-β pathway inducers, including retinoic acid-inducible protein I, mitochondrial antiviral signaling protein, inhibitor of κB kinase ε, and TANK-binding kinase 1. In addition, NP1 interfered with IRF-3–responsive PRD(III-I) promoter activated by SeV and a constitutively active mutant of IRF-3 (IRF-3/SD). Although NP1 suppressed the IRF-3 pathway, it did not affect IRF-3 activation processes, including phosphorylation, dimerization, and nuclear translocation. Coimmunoprecipitation assays confirmed the interaction between NP1 and IRF-3. Additional deletion mutagenesis and coimmunoprecipitation assays revealed that NP1 bound to the DNA-binding domain of IRF-3, resulting in the interruption of an association between IRF-3 and IFNB promoter. Altogether, our results indicate that HBoV NP1 blocks IFN production through a unique mechanism. To our knowledge, this is the first study to investigate the modulation of innate immunity by HBoV. Our findings suggest a potential immune-evasion mechanism used by HBoV and provide a basis for better understanding HBoV pathogenesis. The Journal of Immunology, 2012, 189: 1144–1153.

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Abbreviations used in this article: Co-IP, coimmunoprecipitation; HA, hemagglutinin; HBoV, human bocavirus; IAD, IFR-association domain; IKK-ε, inhibitor of κB kinase ε; KSHV, Kaposi’s sarcoma-associated herpesvirus; MAVS, mitochondrial antiviral signaling protein; ORF, open reading frame; PAMP, pathogen-associated molecular pattern; poly(dA-dT), poly(deoxyadenylic-thymidylic) acid; PRR, pattern recognition receptor; RIG-I, retinoic acid-inducible protein I; RIG-IN, N-terminal domain of retinoic acid-inducible protein I; RT, room temperature; SeV, Sendai virus; TBK-1, TANK-binding kinase 1.

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with which a given virus overcomes the IFN response may be extremely important for its pathogenesis and host range (34, 35). Strategies used by virus-encoded IFN antagonists include general inhibition of cellular gene expression, sequestration of molecules in the IFN-induction pathway, and cleavage or degradation of innate immune components (reviewed in Refs. 36, 37). Many virus-encoded IFN antagonists were reported to suppress the IRF-3 pathway. Some cause the degradation of IRF-3, whereas most of the others exert their functions through inhibition of IRF-3 activation, including phosphorylation, dimerization, and nuclear translocation (reviewed in Refs. 36, 37). Only a limited number of viral proteins was reported to interfere with the function of activated IRF-3 in the nucleus (38–51).

HBoV is ∼5.3 kb in length (1, 52). A recent study of its gene-expression profiles showed that HBoV generally possesses similar genetic information as do bovine parvovirus and canine minute virus (53, 54). In addition to two structural proteins, VP1 and VP2, HBoV encodes three nonstructural proteins: NS1-70, NS1, and NP1. NS1-70 and NS1 are conserved among the parvovirus family, whereas NP1 is only encoded by Bocavirus. The functions of these nonstructural proteins remain unknown. Because an efficient in vitro infection model of HBoV has not been established, we investigated the roles of a nearly full-length HBoV clone and HBoV proteins in the modulation of innate immunity. In this study, we demonstrated that the nearly full-length HBoV clone inhibited both RNA- and DNA-induced IFN-β production. We further found that NP1 suppressed IFN-β production by targeting the IRF-3–signaling pathway. Of interest, NP1 did not inhibit IRF-3 phosphorylation and nuclear translocation, but rather interacted with the DNA-binding domain of IRF-3 and blocked its association with IFNB promoter. To our knowledge, this is the first study investigating the modulation of innate immune signaling by HBoV proteins. Our findings suggest that inhibition of IFN production by NP1 may contribute to the replication and pathogenesis of HBoV and concurrent pathogens.

Materials and Methods
Cells and viruses

A549 cells and HEK 293T cells (both from American Type Culture Collection) were cultured in DMEM (Life Technologies) containing 10% heat-inactivated FBS at 37°C with 5% CO₂. Sendai virus (SeV) was propagated in 10-d-old embryonated eggs and titrated by hemagglutination assay using chicken RBCs. HBoV *+* stool samples from young children were collected from Wuhan Children’s Hospital (Wuhan, China).

Plasmids

All primers used for the plasmid constructs are listed in Table I. HBoV1 (isolate WH, GenBank accession number, FJ496754.1, http://www.ncbi.nlm.nih.gov/nuccore/FJ496754.1) genome was isolated from HBoV *+* stool samples. A nearly full-length genome of 5299 nt was amplified by PCR with primers XhoIW1-F and XbaIWHS299-R and cloned into XhoI/XbaI-digested pBluescript SK (+) vector (Stratagene); the resulting clone was named pWH. pWHHA-NS was made by inserting an HA tag into nt 259 at the N terminus of NS1 and NS1-70 open reading frames (ORFs) on pWH with primers HANSoverlap-F and HANSoverlap-R. pWH2C417AHA-NS was generated by mutating nt 2417 from C to A on pWHHA-NS with primers C2417A-F and C2417A-R, resulting in a termination codon in NP1 ORF.

ORFs encoding HBoV proteins with a C-terminal hemagglutinin (HA) tag were amplified with primers NS1-F and NS1HA-R for NS1, NP1-F and NP1HA-R for NP1, VP1-F and VP2HA-R for VP1, and VP2-F and VP2HA-R for VP2. PCR products were cloned into pcCAGGS, and the resulting expression plasmids were named pCA-NS1HA, pCA-NP1HA, pCA-VP1HA, and pCA-VP2HA, respectively. IRF-3 and IRF-3SD (constitutively active mutant of IRF-3) expression plasmids pRES-hrGFP/IRF-3–Flag and pRES−hrGFP/IRF-3−SD/Flag were kindly provided by Dr. Y. L. Lin (55). IRF-3 truncations IRF-3–1–357 (residues 1–357, primers IRF-3–F and 1-357IRF-3–R), IRF-3–1–240 (residues 1–240, primers IRF-3–F and 1-240IRF-3–R), and IRF-3–1–197 (residues 1–197, primers IRF-3–F and 1-197IRF-3–R) were constructed by PCR and cloned into the pRES−hrGFP–IRES expression vector (Stratagene). All constructs were verified by DNA sequencing.

The reporter plasmid p25-Luc (p-IFN-β–Luc) was described previously (56, 57). The internal control plasmid pRL-TK was purchased from Promega. pEF–FlagRIG-IN encoding the N-terminal domain of RIG-I (RIG-IN) (a carboxy-terminally truncated, constitutively active RIG-I mutant) was kindly provided by Dr. T. Fujita (Kyoto University, Kyoto, Japan) (58). Expression plasmids pcDNA3-TBK1 and pcDNA3–IKK-ε were gifts from Dr. K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA) (59). The reporter plasmid PRD(III-J–Luc) was kindly provided by Dr. S. Ludwig (University of Muenster, Muenster, Germany) (60).

Transfection and luciferase reporter assay

A549 cells were transfected using Lipofectamine LTX reagent (Invitrogen), and 293T cells were transfected using calcium phosphate reagents of Profection (Promega), according to the manufacturers’ instructions. For

### Table I. Primers used for plasmid constructs

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'–3')</th>
<th>Cloning Site</th>
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<tr>
<td>XhoIW1-F</td>
<td>GCGC TCGAGGCCGAGACATATTGGAGTTC</td>
<td>XhoI</td>
</tr>
<tr>
<td>XbaIWHS299-R</td>
<td>GCGTCCTAGATGTTACAAACACACACTATTAAAGATATAAG</td>
<td>XbaI</td>
</tr>
<tr>
<td>HANSoverlap-F</td>
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</tr>
<tr>
<td>HANSoverlap-R</td>
<td>GCCACCTAGGACAGATGCTGTTATGGATGTTATGAGTACGCTGCTGCTG</td>
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<tr>
<td>C2417A-F</td>
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<td></td>
</tr>
<tr>
<td>C2417A-R</td>
<td>CGACTGGCTGTTACCTATGTCCTGTTACCTTGGGATGCTG</td>
<td></td>
</tr>
<tr>
<td>NS1-F</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>GCCGCGGAGACATATTGGAGTTC</td>
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<tr>
<td>VP1-F</td>
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<td></td>
</tr>
<tr>
<td>VP2-F</td>
<td>GCCGCGGAGACATATTGGAGTTC</td>
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<tr>
<td>VP2HA-R</td>
<td>GCCGCGGAGACATATTGGAGTTC</td>
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<tr>
<td>IRF-3-F</td>
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<td></td>
</tr>
<tr>
<td>357IRF-3–R</td>
<td>GAATCTCGAGCTTTAGGTCCTG</td>
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<tr>
<td>240IRF-3–R</td>
<td>GAATCTCGAGCTTTAGGTCCTG</td>
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<td>197IRF-3–R</td>
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<td>56IRF-3–F</td>
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<tr>
<td>112IRF-3–F</td>
<td>GAATCTCGAGCTTTAGGTCCTG</td>
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*Site for cloning is in bold type. F, Forward; R, reverse.
luciferase reporter assays with SeV and poly(dA-dT) stimulation, 293T cells in 24-well plates were cotransfected with 125 ng reporter plasmid pIFN–β-Luc or PRD(III-I)-Luc, 25 ng internal control plasmid pRL-TK, 500 ng empty vector, HBoV1 nearly full-length, or the indicated amount of HBoV protein-expression plasmids. The total amount of DNA was kept constant by adding empty control plasmid. Twenty-four hours posttransfection, cells were stimulated with 100 HA U ml⁻¹ SeV for 16 h or transfected with 2.5 μg poly(dA-dT) (Sigma-Aldrich) using Lipofectamine 2000 (Invitrogen) for 24 h. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. For assays with stimulation of RIG-IN, MAVS, TBK-1, IKK-ε, or IRF-3/5D, 293T cells were cotransfected with 25 ng empty vector or plasmids encoding these stimulators, 125 ng pIFN–β-Luc or PRD(III-I)-Luc, 25 ng pRL-TK, 500 ng empty vector, or the indicated amount of NP1 expression plasmid. Luciferase activities were measured 36 h posttransfection. Each transfection was performed in duplicate, and three independent experiments were performed. Ratios of firefly luciferase activity/Renilla luciferase activity were normalized to that of cells cotransfected with empty vector, pIFN–β-Luc or PRD(III-I)-Luc, and pRL-TK, without stimulation. Fold change values were determined, and the results are shown as mean ± SD.

Protein extraction, immunoblot analysis, and coimmunoprecipitation assay
Whole-cell lysates were generated using Western blot and immunoprecipitation cell lysis buffer (Beyotime Institute of Biotechnology, Nantong, China), according to the manufacturer’s instructions. Immunoblots were performed, as described previously (61). The following primary Abs were used: rabbit anti-HA (Beijing CoWin Biotech, Beijing, China) and mouse anti-HA (Ab-mart), or anti-Flag (Bioss, Beijing, China) for Flag-tagged proteins from coimmunoprecipitation (Co-IP) assays; rabbit anti-IRF-3 (PROTEINTECH group) for endogenous IRF-3; rabbit anti–p–IRF-3 (Ser396; Cell Signaling Technology) for p-IRF-3; mouse anti–β-actin (PROTEINTECH group) for loading control β-actin; rabbit anti-HDAC1 (Beyotime Institute of Biotechnology) for nuclear extracts loading control HDAC1; rabbit anti-α-tubulin (Anbo, Changzhou, China) for loading control tubulin of cellular extracts; rabbit anti-NP1 (raised against purified full-length NP1 protein) for HBoV1 NP1; and rabbit anti-VP2 (raised against purified full-length VP2 protein) for HBoV1 VP1 and VP2. HRP-conjugated secondary Ab and Super Signal West Pico Chemiluminescent Substrate (Thermo) were used, and signal was detected using a Fluochern HD2 Imaging System (Alpha Innotech, San Leandro, CA).

For Co-IP assays, 293T cells in a six-well plate were cotransfected with the indicated expression plasmids or empty vectors (two wells/sample). In some cases, cells were mock infected or infected with SeV (100 HA U ml⁻¹) at 24 h posttransfection for 16 h. Cells were lysed in 300 μl lysis buffer and clarified by centrifugation at 16,000 × g for 5 min at 4 ˚C. Co-IP assays were performed using a Dynabeads Protein G Immunoprecipitation Kit (Invitrogen). Briefly, 10 μg anti-Flag M2, mouse anti-HA (Ab-mart), or mouse IgG was incubated with 50 μl Dynabeads for 10 min to allow formation of a Dynabeads–Ab complex. After washing, the Dynabeads–Ab complex was incubated with cell lysates for 10 min to allow formation of a Dynabeads–Ab–Ag complex. After four washes, the captured Ags were eluted and subjected to SDS-PAGE and immunoblot analysis.

Immunofluorescence analysis
For the immunofluorescence assay to detect the cellular location of NP1 and IRF-3, A549 cells grown on glass slides were cotransfected with pCA-NP1HA or empty vector. At 24 h posttransfection, cells were mock infected or infected with SeV (100 HA U ml⁻¹) for 8 h, fixed in 4% paraformaldehyde for 10 min at room temperature (RT), and permeabilized with 0.2% Triton X-100 for 15 min at RT. After three rinses with PBS, cells were incubated in blocking solution (PBS supplemented with 3% BSA and 5% normal goat serum) overnight at 4˚C and then incubated with mouse anti-HA Ab (Sigma-Aldrich) and rabbit anti–IRF-3 (PROTEINTECH group) for 1 h at RT. After three rinses with PBS, cells were incubated with Texas Red-conjugated goat anti-mouse IgG (Pierce) and FITC-conjugated goat anti-rabbit IgG (Pierce) for 1 h at RT. After three rinses with PBS, cell nuclei were stained with Hoechst 33258. Cells were analyzed using a TCS SP2 laser scanning confocal microscope (Leica, Wetzlar, Germany) equipped with a cooled CCD camera.

DNA affinity-binding assay
The DNA affinity-binding assay was performed, as described previously, with modification (62). Briefly, a biotinylated oligonucleotide containing the IFNB promoter sequence from −105 to −50 (5'-ATGTAAATGACA-3') was used. The DNA affinity-binding assay was performed as described above, with modification (62). Briefly, a DNA fragment containing the IFNB promoter sequence from −105 to −50 (5'-ATGTAAATGACA-3') was used. The DNA affinity-binding assay was performed as described above, with modification (62). Briefly, a DNA fragment containing the IFNB promoter sequence from −105 to −50 (5'-ATGTAAATGACA-3') was used. The DNA affinity-binding assay was performed as described above, with modification (62). Briefly, a DNA fragment containing the IFNB promoter sequence from −105 to −50 (5'-ATGTAAATGACA-3') was used.

**FIGURE 1.** The nearly full-length clone of HBoV inhibits IFN-β production. (A) Schematic diagram of HBoV genome and the construction of the nearly full-length clone. ORFs are shown in the lower panel. Sites for nearly full-length cloning are shown at the top of the panel. HA tags at the N termini of NS1 and NS1-70 are indicated by a “diamond” symbol. (B and C) 293T cells were cotransfected with pIFN–β-Luc, pRL-TK, and the nearly full-length clone pWHHA-NS or empty vector pBluescript SK (+) (p) for 24 h. Cells were then infected or mock infected with SeV for 16 h (B) or transfected or mock transfected with poly(dA-dT) for 24 h (C). Reporter activity was determined by dual-luciferase reporter assays. The resultant ratios were normalized to fold change value by that of cells cotransfected with empty vector, pIFN–β-Luc, and pRL-TK without SeV infection or poly(dA-dT) transfection. Data shown are representative of three independent experiments, with each determination performed in duplicate (mean ± SD of fold change). (D) Expression of HBoV proteins was monitored by immunoblotting using mouse anti-HA tag for HA-NS1 and HA-NS1–70, rabbit anti-VP2 for VP2, and rabbit anti-NP1 for NP1; β-actin was used as a loading control. One of three experiments is shown. **p < 0.01, Student t test.
TAGGAAAACGTGAAAGGAGAATGCGAATCCCTGTGA-3') was annealed with the corresponding antisense oligonucleotide in 1× saline-sodium citrate buffer (150 mM NaCl, 15 mM sodium citrate). Nuclear extracts were isolated using a Nuclear and Cytoplasmic Protein Extraction Kit, according to the manufacturer's instructions, and protein content was determined using the BCA protein assay kit (both from Beyotime Institute of Biotechnology). A total of 6 pmol biotinylated DNA oligonucleotides was mixed with 200 µg nuclear extracts in 500 µl binding buffer containing 20 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM DTT, and 5 mg/ml BSA, in the presence of 13% glycerol and 20 µg poly(deoxyinosinico-deoxycytidyllic) acid, and incubated for 25 min at RT. Fifty microliters of Streptavidin M280 magnetic beads (Invitrogen), which was washed three times with 400 µl binding buffer, was added to the reaction mixture and incubated for 30 min at 4°C and for 10 min at RT with rotation. The beads were collected and washed three times with 500 µl binding buffer. The bound proteins were eluted by boiling in Laemmli sample buffer and resolved by 10% SDS-PAGE, followed by immunoblotting using mouse anti-Flag tag for bound and input Flag-tagged IRF-3. Equal amounts of nuclear extracts used in the assay were confirmed by immunoblotting using anti-HDAC1 Ab.

Results

Nearly full-length clone of HBoV inhibits IFN-β production

Little is known regarding the interaction between HBoV and human immune responses. In the current study, we focused on the modulation of innate immunity by HBoV. Because an efficient HBoV infection model has not been established, we constructed an HBoV plasmid (pWHHA-NS) containing the HBoV coding sequence (nt 1–5299) (Fig. 1A). We further evaluated the inhibitory activity of this nearly full-length clone on IFN-β production using an IFNB promoter reporter system. Reporter plasmid pIFN–β-Luc and internal control plasmid pRL-TK, together with pWHHA-NS or empty vector, were cotransfected into 293T cells. Twenty-four hours posttransfection, SeV infection and poly(dA-dT) transfection were used as RNA and DNA stimulation, respectively, to promote IFN production. We found that the nearly full-length HBoV clone significantly inhibited both SeV- and poly(dA-dT)–stimulated IFNB promoter activity (Fig. 1B, 1C). Expression of HBoV-specific proteins was confirmed by immunoblot analysis. The structural protein VP1, which is almost identical in sequence to VP2 and only differs in the short N-terminal extension, was not detectable (Fig. 1D).

HBoV NP1 inhibits both SeV- and poly(dA-dT)–stimulated IFN-β production

To determine which protein (or proteins) contribute to the IFN-β inhibitory activity of the nearly full-length HBoV clone, we constructed mammalian expression plasmids of HBoV proteins and evaluated their roles in the modulation of IFN-β production using the IFNB promoter reporter system. We found that the nonstructural proteins NP1 and NS1 significantly inhibited both SeV- and poly(dA-dT)–stimulated IFNB promoter activity; the inhibitory activity of NS1 on poly(dA-dT)–stimulated IFNB promoter activity was relatively moderate compared with that of NP1 (Fig. 2). Of interest, NS1-70, which is a relative small isoform of NS1 lacking the C terminus, showed no inhibitory activity on IFN-β production (Fig. 2). In contrast to NP1 and NS1, the structural protein VP2 enhanced both SeV- and poly(dA-dT)–induced IFN-β production (Fig. 2). The other structural protein (VP1) was not detectable using immunoblot and did not have a significant impact on IFN-β production (data not shown).

Although the overexpression of NP1 and NS1 significantly inhibited IFN-β production, research on the HBoV gene-expression profile showed that NP1 was much more abundant than was NS1 (53). Therefore, we focused on NP1 in the following experiment. To address whether NP1 accounted for the inhibitory activity of the nearly full-length HBoV clone (pWHHA-NS) on IFN-β production, we constructed a NP1-deleted nearly full-length HBoV clone. Because NS1 and NP1 share a partial coding sequence within different reading frames, the complete deletion of NP1 would damage NS1 (Fig. 1A). Instead, we introduced a point mutation (pWHC2417AHA-NS), which was a termination code mutation for NP1 but a silent mutation for NS1 (Fig. 3A). As expected, NP1 was not expressed when pWHC2417AHA-NS was transfected into 293T cells (Fig. 3B). To our surprise, VP2 also was not detectable (Fig. 3B). Because the C2417A mutation was not located within the VP2 ORF, the lack of, or severe reduction in, VP2 protein expression might be caused by the absence of NP1. To test this speculation, we introduced NP1 by cotransfection of pWHC2417AHA-NS and NP1 expression plasmid into 293T cells. We then transfected or mock infected by SeV for 16 h (A) or transfected or mock transfected with poly(dA-dT) for 24 h (B). Reporter activity was determined by dual-luciferase reporter assays. The resultant ratios were normalized to fold change value by that of cells cotransfected with empty vector, pIFN–β–Luc and pRL-TK without SeV infection, or poly(dA-dT) transfection. Data shown are representative of three independent experiments, with each determination performed in duplicate (mean ± SD of fold change).

Expression of HBoV proteins was monitored by immunoblotting using rabbit anti-HA tag; β-actin was used as a loading control (lower panel). One of three experiments is shown. *p < 0.05, **p < 0.01, Student t test.
promoter reporter assay to verify the role of NP1 at the level of a nearly full-length HBoV clone. As shown in Fig. 3C–E, the inhibitory activities of both pWHC2417AHA-NS and pWHC2417AHA-NS plus the VP2 expression plasmid were much weaker than that of pWHHA-NS in response to either SeV or poly(dA-dT) stimulation, indicating that NP1 contributed to the efficient inhibition of IFN-β production by the nearly full-length HBoV. Taken together, we identified NP1 as an IFN antagonist inhibiting both SeV- and DNA-induced IFN-β production.

**HBoV NP1 interferes with IRF-3 pathway**

To understand the mechanism by which NP1 inhibits IFN-β expression, plasmids expressing IFN-inducing molecules in the IFN-β–production pathway, including RIG-IN, MAVS, TBK-1, or IKK-ε, were transfected into 293T cells together with pIFN-Luc, pRL-TK, NP1 expression plasmid, or empty vector. As shown in Fig. 4, expression of RIG-IN, MAVS, TBK-1, or IKK-ε efficiently induced the IFNB promoter activity. In contrast, in the presence of NP1 expression plasmid, the activity of the IFNB promoter was substantially inhibited in a dose-dependent manner. In the presence of 500 ng NP1 expression plasmid (per well in a 24-well plate), the inhibitory activity was ~93% for RIG-IN, 82% for MAVS, 94% for TBK-1, and 90% for IKK-ε, indicating that NP1 interferes with the function of these inducers (Fig. 4).

IRF-3 is a key transcription activator in the IFN-β–production pathway. SeV infection, RIG-IN, or MAVS expression activates downstream kinases TBK-1 and IKK-ε, which phosphorylate IRF-3, leading to its activation. We hypothesized that NP1 probably inhibited the IRF-3–mediated pathway, resulting in the reduction of IFN-β production. To test this hypothesis, we transfected 293T cells with reporter plasmid PRD(III-I)–Luc containing the IRF-3–responsive PRD(III-I) region in the IFNB promoter, the internal control plasmid pRL-TK, and increasing amounts of NP1 expression plasmid for 24 h, followed by infection or mock infection with SeV for 16 h. As seen in Fig. 5A, SeV infection induced PRD(III-I) promoter activity by 8-fold, whereas NP1 inhibited PRD(III-I) promoter activity in a dose-dependent manner. In the presence of 500 ng NP1 expression plasmid, the inhibitory activity was ~68%. It is noteworthy that NP1 also inhibited the induction activity of IRF-3/5D, a constitutively activated form of IRF-3 (Fig. 5B), indicating that NP1 inhibits IFN-β production downstream of IRF-3 activation.

**HBoV NP1 does not suppress IRF-3 phosphorylation and nuclear translocation**

Upon stimulation, IRF-3 can be activated and then translocated into the nucleus. We next addressed whether NP1 interfered with these processes. The NP1 expression plasmid or empty vector was

![FIGURE 3. NP1-deleted nearly full-length HBoV clone exhibits significantly decreased inhibition of IFN-β production. (A) Strategy for construction of NP1-deleted nearly full-length clone. ORF NS1 and NP1 share sequence nt 2410–2666 with different reading frames. Codons are shown as three-letter segments. C2417A mutation is framed and indicated by a “star” symbol. (B) NP1 deletion eliminates VP2 expression. 293T cells in 35-mm dishes were transfected with 1 μg of the indicated nearly full-length clones, together with 2 μg NP1 expression plasmid, or empty vector. Forty-eight hours post-transfection, cells were harvested. HBoV proteins were monitored by immunoblotting using mouse anti-HA tag for HA-NS1 and HA-NS1-70, rabbit anti-VP2 for VP2, and rabbit anti-NP1 for NP1 and NP1-HA; β-actin was used as a loading control. One of three experiments is shown. (C) and (D) 293T cells in 24-well plates were cotransfected with pIFN-β-Luc, pRL-TK and the nearly full-length clone pWHHA-NS, NP1-deleted nearly full-length clone pWHC2417AHA-NS, or empty vector pBluescript SK (+) for 24 h. Cells were then infected or mock infected by SeV for 16 h (C) or transfected or mock transfected with poly(dA-dT) for 24 h. Reporter activity was determined by dual-luciferase reporter assays. The resultant ratios were normalized to fold change value by that of cells cotransfected with empty vector, pIFN-β–Luc and pRL-TK without SeV infection, or poly(dA-dT) transfection (D). Data shown are representative of three independent experiments, with each determination performed in duplicate (mean ± SD of fold change). (E) Expression of HBoV proteins in (C, D) was monitored by immunoblotting, as described in (B). One of three experiments is shown. **p < 0.01, Student t test.
transfected into 293T cells. Twenty-four hours posttransfection, cells were infected with SeV for 8 h. SeV infection induced IRF-3 phosphorylation (Fig. 6A, lane 2). Of interest, overexpression of NP1 did not inhibit IRF-3 phosphorylation (Fig. 6A, lane 3). To further test whether NP1 interferes with the nuclear translocation of IRF-3, 293T cells and A549 cells were transfected with the NP1 expression plasmid or empty vector for 24 h, followed by infection or mock infection with SeV for 8 h. Cytoplasmic proteins and nuclear proteins were subsequently prepared from 293T cells, followed by immunoblotting analysis, to determine the distribution of IRF-3. For A549 cells, immunofluorescence assays were performed to examine the location of IRF-3. As shown in Fig. 6B and 6C, SeV infection induced the nuclear translocation of IRF-3, whereas NP1 had a negligible effect on the location of IRF-3, indicating that NP1 does not inhibit IRF-3 nuclear translocation upon SeV infection.

**HBoV NP1 interacts with IRF-3**

Because NP1 interfered with the IRF-3 pathway but did not inhibit IRF-3 activation and nuclear translocation, we further investigated the underlying mechanisms. A previous report (53) and the results of this study (Fig. 6B, 6C) showed that NP1 mostly located in the nucleus. Because activated IRF-3 is also localized in the nucleus, NP1 may interfere with the function of IRF-3 in the nucleus through direct binding. We assessed the interaction between NP1 and IRF-3. NP1-HA and IRF-3–Flag (or IRF-3/5D–Flag) expression plasmids were cotransfected into 293T cells. Twenty-four hours posttransfection, cells were infected or mock infected with SeV for 8 h. Co-IP assays were performed to determine the interaction between NP1 and IRF-3 or IRF-3/5D. As shown in Fig. 7, NP1-HA was immunoprecipitated together with IRF-3–Flag and IRF-3/5D–Flag using anti-Flag tag (upper panel). Similar results were observed in reciprocal Co-IP assays using anti-HA tag (middle panel), suggesting that NP1 interacted with both IRF-3 and the constitutively active mutant IRF-3/5D. In addition, p-IRF-3 was detected in Co-IP products using anti-HA tag (lower panel), indicating that NP1 interacts with p-IRF-3. Given that NP1 localizes almost exclusively in the nucleus (Fig. 4B, 4C), it is likely that NP1 could interact with activated IRF-3 in the nucleus.

**HBoV NP1 binds to IRF-3 DNA-binding domain and blocks the association of IRF-3 with IFNB promoter**

IRF-3 consists of 427 aa, including a DNA-binding domain (aa 1–112) responsible for DNA binding and a IRF-association domain (IAD; aa 197–394) responsible for IRF-3 phosphorylation, dimerization, and interaction with CBP/p300 coactivators. To map the region in IRF-3 responsible for NP1 binding, a series of IRF-3–deletion mutants was constructed (Fig. 8A), and Co-IP assays were performed. Deletion of part (IRF-3 1–357, IRF-3 1–240) or deletion of part (IRF-3 1–357, IRF-3 1–240) of the entire DNA-binding domain (IRF-3 56–427, IRF-3 112–427)
abolished the interaction between IRF-3 and NP1, indicating that the DNA-binding domain of IRF-3 is responsible for NP1 binding. Because the DNA-binding domain is crucial for the association between IRF-3 and the IFNB promoter, NP1 binding to this domain probably interrupted the association of IRF-3 with its responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA. To test this hypothesis, a DNA-binding affinity assay, using dsDNA oligo containing the responsive DNA.

Discussion
HBoV has been identified as a global viral pathogen infecting young children. However, the pathogenesis of HBoV remains to be fully addressed. Interaction between viruses and host innate immune responses is crucial for viral replication. Because of the lack of an efficient in vitro HBoV culture system and an animal model, in the current study, we focused on the modulation of innate immunity by a nearly full-length HBoV clone and HBoV-encoded proteins. We found that the nearly full-length HBoV clone severely inhibited IFN-β production. We further identified the nonstructural protein NP1, a Bocavirus-specific protein, as an antagonist of IFN-β production by modulating the IRF-3 pathway. Of interest, NP1 did not inhibit IRF-3 activation or promote its degradation, as commonly exploited by other IRF-3 antagonists. Rather, NP1 suppressed IFN via a unique mechanism by binding to the DNA-binding domain of IRF-3 after its activation, resulting in a blockade of the association between IRF-3 and the IFNB promoter. Our study reveals a potential mechanism by which HBoV counteracts human innate immune responses.

The interaction between parvoviruses and host immune responses has not been well characterized. Several parvoviruses, including minute virus of mice (63, 64), H-1 parvovirus (63), adenov-associated virus types 2 and 5 (63), mink parvovirus (65), and Kilham rat virus (66), were reported to be inefficient or unable to induce IFN. A recent study of MVMP suggests that the failure to produce IFN in normal cells is likely due to the lack of cellular sensors for recognizing MVMP or its products during replication (67). Research on mink parvovirus showed that pre-existing infection of this virus did not inhibit IFN production induced by
inducers, including RIG-IN, MAVS, TBK-1, and IKK-ε. Given that NP1 is abundantly expressed during HBoV infection (53), HBoV NP1 probably functions as an IFN antagonist, evading innate immune responses during HBoV infection.

NP1 protein is only encoded by Bocavirus, implying that HBoV may use distinctive mechanisms to modulate innate immunity compared with other paroviruses not belonging to this genus. Meanwhile, it is likely that NP1 proteins of other bocaviruses exhibit similar function. Indeed, although beyond the scope of this study, we found that the NP1 proteins of HBoV2 and HBoV3 inhibited IFN-β production, and the inhibitory activities were comparable to those of HBoV1 (Z. Zhang and H. Wang, unpublished observations). It is currently under investigation whether NP1 proteins of HBoV4 and animal bocaviruses can inhibit IFN production. Although NP1 severely blocked IFN-β production, NP1 deletion did not completely eliminate the inhibitory activity of the nearly full-length HBoV clone on IFN-β production, indicating that HBoV protein(s) other than NP1 might also interfere with IFN-β production. In agreement, we found that the newly identified HBoV nonstructural protein NS1, which is conserved among the parovirus family, also inhibited IFN production when it was overexpressed. The significance of HBoV NS1 and its homologs of other paroviruses on innate immune modulation and the underlying mechanisms remain to be addressed.

IRF-3 is a key transcription activator for IFN production. Recognition of many types of PAMPs of invading pathogens, such as dsRNA, ssRNA, cytoplasmic DNA, and viral proteins, leads to the activation of IRF-3, and the PRRs involved include TLRs, RIG-I, melanoma differentiation-associated protein 5, and the newly identified DNA receptor Z-DNA–binding protein 1 (reviewed in Ref. 36). Although PAMP(s) recognized during HBoV infection are unknown, NP1 likely blocks signal transduction upon recognition of the PAMP(s) by modulating IRF-3, leading to the interruption of IFN production. Because other pathogens frequently coinfect children during HBoV infection, it is reasonable to infer that HBoV NP1 may assist these pathogens in counteracting IFN production and, thus, enhance the outcome of coinfection.

Although HBoV NP1 interferes with the IRF-3 pathway, it does not inhibit IRF-3 activation; the interference occurs through suppressing the function of activated IRF-3 in the nucleus. Several viral proteins were also reported to modulate the function of activated IRF-3. For instance, ICP0 of bovine herpes virus 1, ORF 61 homologs of other parvoviruses on innate immune modulation and the underlying mechanisms remain to be addressed.

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ICPF of HSV-1 blocks the association of IRF-3 with the IFNB promoter by recruitment of IRF-3 and CBP/p300 to nucleoli structures (50); and K-bZIP and LAN-1 of KSHV bind to IRF-3-responsive elements, which block the association of IRF-3 with the IFNB promoter in a competing manner (47, 48). In the current study, overexpression of HBoV NP1 neither significantly affected the levels of IRF-3 and IRF-3 activation nor the localization of IRF-3 in the nucleus. Although it is unknown whether NP1 interferes with the recruitment of CBP/p300 coactivators by IRF-3, NP1 clearly blocks the association of IRF-3 with the IFNB promoter. Unlike K-bZIP and LAN-1 of KSHV, NP1 exerts its function by interacting with the DNA-binding domain of IRF-3. Among the viral proteins that can bind to IRF-3, HBoV NP1 is the only one targeting the IRF-3 DNA-binding domain. Interaction of HBoV NP1 with the IFNB DNA-binding domain probably disables IRF-3’s access to its responsive elements in the IFNB promoter, thereby interrupting IFN-β transcription.

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Disclosures
The authors have no financial conflicts of interest.

References


