Hemoglobin Subunit Beta Interacts with the Capsid Protein and Antagonizes the Growth of Classical Swine Fever Virus

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The capsid (C) protein of the *Flaviviridae* family members is involved in nucleocapsid formation and virion assembly. However, the influence of C protein-interacting partners on the outcome of pestivirus infections is poorly defined. In this study, hemoglobin subunit beta (HB) was identified as a C protein-binding protein by glutathione S-transferase pulldown and subsequent mass spectrometry analysis of PK-15 cells, which are permissive cells for classical swine fever virus (CSFV). Coimmunoprecipitation and confocal microscopy confirmed that HB interacts and colocalizes with the C protein in the cytoplasm. Silencing of HB with small interfering RNAs promoted CSFV growth and replication, whereas overexpression of HB suppressed CSFV replication and growth. Interestingly, HB was found to interact with retinoic acid-inducible gene I and increase its expression, resulting in increased production of type I interferon (IFN). However, HB was unable to suppress CSFV growth when the RIG-I pathway was blocked. Overall, our results suggest that cellular HB antagonizes CSFV growth and replication by triggering IFN signaling, and might represent a novel antiviral restriction factor. This study reports for the first time the novel role of HB in innate immunity.

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a highly contagious disease of pigs that is responsible for significant economic losses worldwide. CSFV belongs to the genus *Pestivirus* of the family *Flaviviridae*. The genome of CSFV is a single-stranded, positive-sense RNA molecule approximately 12.3 kb in length that encodes a polyprotein that is processed to yield 12 mature proteins (NH₂-N^{pro}-C-E^{gns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) (1). Structural proteins of CSFV consist of glycoproteins E^{gns}, E1, and E2 and capsid (C) protein, a nucleocapsid protein of unknown symmetry (2).

The C protein of pestiviruses is a small, highly basic polypeptide that binds to RNA with low affinity and specificity (3, 4). The C protein of CSFV influences the regulation of cellular transcription (5) and interacts with host SUMOylation proteins (6). The ability of the C protein to bind the cellular IQGAP1 protein during infection plays a critical role in determining the virulence of the virus (7). Recently, some studies showed that the C protein is dispensable for infectious virion formation and virus propagation *in vitro* (8, 9).

Studies of the C protein of hepatitis C virus (HCV), another member of the *Flaviviridae* family, provide further insight into the possible functions of the C protein of CSFV. The HCV C protein has been shown to be crucial for virus particle production because it is the structural component of the viral nucleocapsid and is required for the formation of the active HCV replication/assembly complex in host cells (10, 11). A number of studies have revealed that a variety of host proteins, such as vimentin, interact with the C protein (12, 13) and influence the pathogenesis of HCV by modulating processes such as cell signaling, transformation and proliferation, cellular and viral gene expression, apoptosis, and lipid metabolism (14–19).

Hemoglobin is the metalloprotein responsible for oxygen transport and is present in the red blood cells of all vertebrates (20), except members of the fish family *Channichthyidae* (21). The hemoglobin molecule is composed of four globular protein sub-

units ($\alpha_2\beta_2$), each of which is composed of a protein chain tightly associated with a nonprotein heme group. Each protein chain is arranged as a set of alpha-helix structural segments connected in a structure known as a globin fold arrangement, so called because this folding motif occurs in other heme/globin proteins, such as myoglobin (22). Hemoglobin has an oxygen binding capacity of 1.34 ml of O₂/g of hemoglobin (23), which increases the total blood oxygen capacity 70-fold over that of dissolved oxygen in blood. The mammalian hemoglobin molecule can bind (carry) up to four oxygen molecules at one time (24). Hemoglobin is also involved in the transport of other gases; it carries some of the body's respiratory carbon dioxide (about 10% of the total) as carbamino hemoglobin, as well as the important regulatory molecule nitric oxide, bound to a globin protein thiol group, releasing it at the same time as oxygen (25). Hemoglobin is also found outside red blood cells and their progenitor lines. Other cells that contain hemoglobin include A9 dopaminergic neurons in the substantia nigra, macrophages, alveolar cells, and mesangial cells in the kidney. In these tissues, hemoglobin functions as an antioxidant and a regulator of iron metabolism (26).

To date, some studies have focused on the roles of the structural glycoproteins of CSFV in virus replication and virulence (2, 27, 28), but information on C protein-interacting proteins and their impact on the outcome of CSFV infection is limited. Here, we show that cellular hemoglobin subunit beta (HB) interacts specifically with the CSFV C protein and antagonizes CSFV growth and genomic replication.

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TABLE 1 Primers used in this study

Primer	Sequence (5'–3')	Use
GST-C-F GST-C-R	CGGAATTCATGTCTGATGATGGCGCAAGTG CCGCTCGAGTTAGGCTTCAACTGGTTGATAC	Amplification of C
Myc-C-F Myc-C-R	CGGAATTCATTCTGATGATGGCGCAAGTGG ATCTCGAGTTAGGCTTCAACTGGTTGATAC	Amplification of C
3×Flag-HB-F 3×Flag-HB-R	CGGAATTCATGGTGCATCTGTCTGCTGAG GGGGTACCTTAGTGGTACTTGTGGGCCAGGGC	Amplification of HB
Δ1-50-F Δ1-50-R	CGGAATTCATCCAATGCCGATGCCGTCATG GGGGTACCTTAGTGGTACTTGTGGGCCAGGGC	Amplification of HB (Δ1-50)
Δ50-100-F Δ50-100-R	CATGGCCAAGCGGCGAGCCATGACGGCATCGGCATTGGA TCCAATGCCGATGCCGTCATGGCTCGCCGCTTGCCCATG	Amplification of HB (Δ50-100)
Δ100-146-F Δ100-146-R	CGGAATTCATGGTGCATCTGTCTGCTGAG GGGGTACCTTAATGGCCAAGCGGCGGAGC	Amplification of HB (Δ100-146)
RIG-I-F RIG-I-R	GGCTCGAGCTATGACAGCAGAGCAGCGGGCG TTGCGGCCGCTCACTCAAGGTTGCCCATTC	Amplification of RIG-I
MDA5-F MDA5-R	GGGAATTCCTATGTCGTCGGATGGGTATTC GGCTCGAGTCAGTCCTCATCTAGACAAAC	Amplification of MDA5
IFN-β-F IFN-β-R	GGCTGGAATGAAACCGTCAT TCCAGGATTGTCTCCAGGTCA	Quantitative real-time PCR for detection of IFN-β
GAPDH-F GAPDH-R	ACATGGCCTCCAAGGAGTAAGA GATCGAGTTGGGGCTGTGACT	Quantitative real-time PCR for detection of GAPDH

MATERIALS AND METHODS

Cells, viruses, and virus titer assays. HEK293T cells, SK6 cells, and PK-15 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) known to be free of bovine pestivirus and anti-pestivirus antibodies. CSFV strain Shimen was propagated in CSFV-permissive PK-15 cells and SK6 cells. Virus titers in the culture supernatants of CSFV-infected PK-15 and SK6 cells were determined by the Reed-Muench method (29).

Plasmids. The p3×Flag-HB plasmid encoding the HB protein (GenBank accession no. NM_001144841.1) with a Flag tag at its N terminus was constructed by cloning HB cDNA into a p3×Flag-CMV10 vector (E7658; Sigma) with the EcoRI and KpnI restriction enzymes. The CSFV C protein cDNA (GenBank accession no. AY775178.2) was cloned into the pCMV-Myc vector (Clontech) with the EcoRI and XhoI restriction enzymes to generate the pMyc-C plasmid. Retinoic acid-inducible gene I (RIG-I; GenBank accession no. NM_213804.2) or melanoma differentiation-associated gene 5 (MDA5; GenBank accession no. NM_001100194.1) cDNA was cloned into the pCMV-Myc vector (Clontech) with the NotI and XhoI or EcoRI and XhoI restriction enzymes to generate the pMyc-RIG-I or pMyc-MDA5 plasmid. For bacterial expression of the glutathione S-transferase (GST)-tagged C protein, the C protein gene region was subcloned into the pGEX-6p-1 vector (28-9546-48; GE Healthcare), creating pGEX-C. A series of mutant forms of HB was generated from p3×Flag-HB by conventional PCR with the mutagenesis primers listed in Table 1. All plasmids were verified by sequencing.

Plasmid DNA transfection. Cells in 6- or 12-well plates (Nunc) cultured at 37°C in a humidified incubator with 5% CO₂ were transfected with respective plasmids (4 μg each) with the X-tremeGENE HP DNA transfection reagent (catalog no. 06366236001; Roche) according to the manufacturer's instructions. At 4 h posttransfection (hpt), the transfection mixture was replaced with complete growth medium and incubated for an additional 48 h before being used for assays.

Virus infection and treatment. At 48 h after DNA or small interfering RNA (siRNA) transfection, cells were infected with CSFV strain Shimen at a multiplicity of infection (MOI) of 0.1 or Sendai virus (SeV) at an MOI of 1. After 2 h, the viral inoculum was removed and the infected cells were washed twice with phosphate-buffered saline (PBS; pH 7.4) and refed with DMEM containing 2% FBS. At various time points postinfection, cell-free culture supernatants and cell lysates were harvested and stored at –80°C until use. To examine the effects of beta interferon (IFN-β), SK6 cells were left untreated or treated with IFN-β (1,000 IU/ml) (ab87929; Abcam) at 12 h prior to infection with CSFV, and CSFV titers and genomic replication were analyzed.

GST pulldown assays. For GST pulldown assays, GST or GST-C protein produced in *Escherichia coli* BL21(DE3) cells was conjugated to glutathione beads (10049253; GE Biosciences) and blocked for 1 h in 5% bovine serum albumin. The beads were then washed twice with TIF buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM MgCl₂, 0.1% NP-40, 10% glycerol, 0.1 mM dithiothreitol [DTT], 1 mg/ml protease inhibitor) and incubated for 2 h at 4°C with recombinant Flag-tagged HB harvested from transfected HEK293T cells. The beads were washed at least six times with TIF buffer, followed by elution and detection of the proteins by SDS-PAGE and immunoblotting.

Coimmunoprecipitation (co-IP). HEK293T cells were transfected with the indicated constructs as described above. The transfected cells were harvested at 48 hpt, washed three times with cold PBS (pH 7.4), and lysed with NP-40 buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.5% NP-40, 0.5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml protease inhibitor cocktail (Roche) at 4°C for 1 h. Clarified extracts were precleared with protein A/G beads (SC-2003; Santa Cruz) and then incubated with protein A/G beads plus anti-Flag monoclonal antibody (MAb) F3040 (Sigma) for 4 h. The beads were then washed with NP-40 buffer and boiled in sample buffer, and the proteins were subjected to SDS-PAGE, followed by immunoblotting analysis with

FIG 1 Identification of C-interacting proteins by GST pulldown (A) and expression of HB protein in different swine tissues and cells (B). GST-C protein produced in *E. coli* BL21(DE3) cells was used for a GST pulldown assay. GST-empty vector was used as a negative control. The eluted protein complex was resolved by 12% SDS-PAGE, followed by Coomassie staining, and analyzed by MS. The two bands binding specifically to the C protein are indicated by asterisks. The expression of the HB protein in swine tissues and PBMCs deprived of red blood cells was detected by immunoblotting with anti-HB pAb.

anti-Flag (F7425; Sigma) and anti-Myc polyclonal antibodies (pAb) (E022050-1; Earthox LLC).

Real-time RT-PCR. Following the indicated treatment, total RNA was extracted from CSFV-infected cells with TRIzol (15596026; Invitrogen) and treated with DNase I to remove potential genomic DNA contamination. The isolated RNA was then reverse transcribed to cDNA with Moloney murine leukemia virus reverse transcriptase (TaKaRa) according to the manufacturer's instructions. Quantification of numbers of genomic copies of CSFV was performed by a previously described quantitative real-time reverse transcription (RT)-PCR assay (30).

Gene expression was quantified by real-time PCR with SYBR Permex *Ex Taq* II (DRR081A; TaKaRa) in the LightCycler 480 II real-time PCR system (Roche). The primers used to detect IFN- β are listed in Table 1. The relative abundance of each target was obtained by normalization with endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

RNA interference. siRNAs targeting HB or RIG-I were used at a final concentration of 400 or 200 nM, unless otherwise stated. Cells were transfected with siRNAs with X-tremeGENE siRNA transfection reagent (4476093001; Roche) as described previously (31). The siRNA target sequences of HB were GGACGAAGTTGGTGGTGAG (siHB-1) and GGTGCATCTGTCTGCTGAG (siHB-2). The siRNA target sequences of RIG-I were GGTACAAAGTTGCAGGCA (siRIG-I-1), GCAAACAGCATCCTTATAA (siRIG-I-2), and CCATAACTCTTGGAGGCTT (siRIG-I-3). Western blotting was used to analyze endogenous swine RIG-I protein production with goat anti-RIG-I pAb SC-48932 (Santa Cruz).

Confocal imaging. HEK293T cells were cotransfected with pMyc-C (2 μ g) and p3 $\times$ Flag-HB (2 μ g). After 48 h of incubation, cells transfected or infected with CSFV were fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.1% Triton X-100 for 15 min. The cells were then incubated with anti-Myc MAb E022050-1 (Earthox) or anti-C MAb (32) for 2 h and then incubated for 2 h with anti-Flag pAb F7425 (Sigma) or anti-HB pAb SC-22718 (Santa Cruz). The cells were then incubated with goat anti-mouse IgG (whole molecule)-fluorescein isothiocyanate antibody (F2012; Sigma) and goat anti-rabbit IgG (whole molecule)-tetramethyl rhodamine isocyanate (TRITC) antibody (T6778; Sigma) or an anti-goat IgG (whole molecule)-TRITC antibody (T7028; Sigma). Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 15 min and examined with a Leica SP2 confocal system (Leica Microsystems). Signal colocalization was analyzed with the program Colocalizer Pro (Colocalization Research Software, Boise, ID).

Luciferase (Luc) reporter assay. HEK293T cells (10^5) in 24-well plates were transfected with 500 ng of pIFN- β -Luc, 10 ng of pRL-SV40-Renilla (Promega) as an internal control, and 1 μ g of each of the indicated expression vectors. After 24 h, cells were transfected with or without 1.0 μ g of poly(I-C). PK-15 cells (10^5) in 24-well plates were transfected with 1 μ g of pIFN- β -Luc, 20 ng of pRL-SV40-Renilla as an internal control, and 400 nM siRNAs targeting HB (siHB). After 24 h, cells were infected with CSFV or SeV for 48 h. Reporter gene activity was analyzed with the Dual-Luciferase Reporter 1000 assay system (Promega) and measured with a TD-20/20 Luminometer (Turner Designs) according to the manufacturer's

TABLE 2 Proteins present in the two specific bands observed in the GST-C lane of Fig. 1 as identified by MS

Band and accession no.	Protein name (approx mol mass [kDa])	Protein score confidence interval (%)
1		
gi 5542423	Chain B, crystal structure determination of porcine hemoglobin at 1.8 Å resolution (16,082.4)	98.432
gi 122784	Hemoglobin subunit theta (16,027.3)	97.336
2		
gi 4929901	Nuclear matrix-targeting signal of transcription factor Aml-1 (32,288.3)	100
gi 545835	Phospholipase C β 4 (21,399.8)	88.01

FIG 2 CSFV C protein interacts with HB. (A) Co-IP of HB with C protein. HEK293T cells were cotransfected with the indicated plasmids (+) or empty vectors (–), and the whole-cell lysates obtained at 48 hpt were immunoprecipitated (IP) with anti-Flag MAb. After separation by SDS-PAGE, proteins were detected by immunoblotting with the indicated antibodies. A 5% aliquot of whole-cell lysate was also probed to confirm protein expression. The identities of the protein bands are indicated on the right. The asterisk indicates a nonspecific band. (B) GST pulldown assay. Glutathione beads conjugated to GST or the GST-C protein fusion protein were incubated with recombinant HB. After washing, proteins were eluted from the beads and SDS-PAGE was performed. The presence of HB was detected by immunoblotting with anti-Flag MAb. GST and GST-C protein expression was confirmed by immunoblotting with rabbit anti-GST pAb. (C) Co-IP of CSFV C protein with endogenous HB. CSFV-infected (+) or mock-infected (–) PK-15 cells were used for IP with anti-C protein MAb and immunoblotted with the indicated antibodies. The identities of the bands are shown on the right. (D) Colocalization of C protein with HB. HEK293T cells were cotransfected with p3×Flag-HB and pMyc-C. Cells were fixed at 48 hpt and subjected to indirect immunofluorescence to detect Myc-C (green) and 3×Flag-HB (red) with mouse anti-Myc and rabbit anti-Flag antibodies. The position of the nucleus is indicated by DAPI (blue) staining in the merged image. (E) Colocalization of C protein with endogenous HB. PK-15 cells were mock infected or infected with CSFV strain Shimem. Cells were fixed at 48 h postinfection and subjected to indirect immunofluorescence to detect C protein (green) and HB (red) with mouse anti-C protein and goat anti-HB antibodies. The position of the nucleus is indicated by DAPI (blue) staining in the merged image.

instructions. Three independent experiments were carried out in duplicate. Error bars represent standard deviations from the mean.

Protein extraction from swine tissues and isolation of porcine peripheral blood mononuclear cells (PBMCs). Tissues were collected from 3-month-old healthy crossbred pigs and stored at -80°C . The experimental and animal handling protocols were approved by the Ethics Committee on Experimental Animal Usage and Animal Welfare, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Frozen tissues were broken into small pieces and washed with PBS to remove blood. The tissue pieces were then ground into homogenate with a mortar and pestle. The tissues were washed three times with ice-cold PBS and lysed with red blood cell lysis buffer (C3702; Beyotime) for 30 min at 4°C to remove red blood cells. The lysates were then clarified by centrifugation at 10,000 rpm for 10 min at 4°C . The tissues were lysed with lysis buffer containing 7 M urea, 2 M thiourea, 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM DTT, 0.2% Bio-Lyte 3/10, and 1 mM PMSF at a volume ratio of 1:10 for 1 h on ice, and the lysates were then clarified by centrifugation at 10,000 rpm for 30 min at 4°C . The supernatants were collected, and the protein concentration was determined by the Bradford assay (PA102; Tiangen).

Heparinized blood was diluted 1:3 in sterile PBS (pH 7.4) and subsequently layered over pig lymphocyte separation medium (LTS1110; TBD-science) according to the manufacturer's protocol. After centrifugation at $2,000 \times g$ for 20 min at room temperature, the mononuclear cell band at the interface was removed and washed twice with basal medium by centrifugation at $2,000 \times g$ for 10 min at room temperature. PBMCs were lysed with red blood cell lysis buffer (C3702; Beyotime) for 30 min at 4°C to remove red blood cells. The lysates were clarified by centrifugation at 10,000 rpm for 10 min at 4°C . PBMCs were lysed with NP-40 buffer for 30 min on ice, and the lysates were clarified by centrifugation at 10,000 rpm for 30 min at 4°C . The supernatants were collected, and the protein concentration was determined as described above.

Statistical analysis. Statistical analyses were performed with SPSS 13.0 software. Student's *t* test or one-way analysis of variance was used to compare viral titers and genomic replication. A *P* value of <0.05 was considered significant.

RESULTS

CSFV C-interacting proteins identified by GST pulldown and MS. To identify cellular proteins that potentially associate with the

FIG 3 The C-terminal portion of HB is required for association with C protein. (A) Schematic representation of the protein domains of swine HB and the individual deletion mutant (Mut) forms of HB tested in this study. (B) Co-IP analysis of the association of Myc-C protein with Flag-tagged HB or mutant forms of HB in HEK293T cells cotransfected with the indicated expression plasmids (+) or empty vectors (–). aa, amino acids.

CSFV C protein in PK-15 cells, GST pulldown was coupled with mass spectrometry (MS). The results showed that several common bands were detected in both the GST and GST-C lanes and at least two specific bands were detected in the GST-C protein lane

(Fig. 1A, indicated with an asterisk). These protein bands were excised and subjected to MS analysis. Several cellular proteins were identified as C protein binding proteins in this analysis, and their identities, including the number of separate peptides de-

FIG 4 Knockdown of HB increases CSFV growth. (A) Knockdown of HB protein levels by siRNA treatment. PK-15 cells transfected with no siRNA (Mock) or scrambled siRNA (siScr), left untreated (No treat), or treated with different concentrations (nM) of siHB (as indicated at the top of each lane) were harvested at 48 hpt. Endogenous HB was detected by immunoblotting with antibodies directed against the indicated proteins. (B) CSFV C protein is increased in HB knockdown cells. PK-15 cells treated with 400 nM siHB, left untreated (No treat), mock treated, or treated with siScr for 48 h were infected with CSFV at 100 tissue culture infective doses (TCID₅₀) for 48 h or 72 h. Endogenous HB was detected by Western blotting with anti-HB pAb, and CSFV C protein was detected by Western blotting with anti-C protein MAb. (C) CSFV titers in HB knockdown cells. siRNA transfection and virus infection were performed as described for panel B, and the virus titers in supernatants collected at 48 and 72 h postinfection were determined by immunofluorescence assay and expressed as TCID₅₀/ml. Error bars represent the standard error of the mean from three independent experiments. P values are indicated above the bars.

FIG 5 Overexpression of HB reduces CSFV growth. (A) Ectopic expression of HB in PK-15 cells. PK-15 cells were transfected with p3xFlag-HB (+) or the empty vector (–) for 12 h and then infected with 100 tissue culture infective doses (TCID_{50}) of CSFV. HB expression was analyzed by Western blotting of cell lysates collected at the indicated time points after infection. β -Tubulin was used as a loading control. (B) Reduced CSFV growth in HB-overexpressing cells. Transfection and infection conditions were as described for panel A, but the titers of virus in the supernatant collected at 48 and 72 h postinfection were determined by the Reed-Muench method (29). Error bars represent the standard errors of the means from three independent experiments. *P* values are indicated above the bars.

ected by matrix-assisted laser desorption ionization–time of flight MS analysis, are shown in Table 2. The present study focused on the cellular protein HB, which was detected in band 1.

To determine the tissue and cell distribution of HB, the expression of swine HB protein was assessed by immunoblotting. The results showed that HB was expressed in all of the swine tissues

and cells examined, including kidney, liver, lung, spleen, and tonsil tissues and PBMCs (Fig. 1B).

HB interacts and colocalizes with C protein. To confirm the interaction between HB and C proteins, co-IP experiments were performed with HEK293T cells transiently coexpressing 3xFlag-tagged HB and Myc-tagged C proteins. Cells coexpressing 3xFlag

FIG 6 HB is involved in CSFV replication, and CSFV inhibits HB expression. (A) Transcription levels after HB knockdown assessed with a quantitative RT-PCR assay at 48 and 72 h after CSFV infection. (B) Transcription levels after HB overexpression were assessed with a quantitative RT-PCR assay at 48 and 72 h after CSFV infection. EV, empty vector. Cells were transfected with control siRNA (siScr) or siRNA targeting HB. β -Tubulin was used as a loading control. Error bars represent the standard error of the mean of three independent experiments. *P* values are indicated above the bars. (C) Expression of endogenous HB protein in PK-15 cells infected with 10^3 tissue culture infective doses (TCID_{50}) of CSFV at various time points.

FIG 7 HB activates the IFN signaling pathway, and IFN- β upregulates HB expression. (A) The effects of transfection with 3 $\times$ Flag-HB and/or Myc-C on the activation of a Luc reporter gene is presented as fold induction relative to samples transfected with the empty vector (EV). Expression of HB and/or C protein increased IFN- β transcription in HEK293T cells transfected with poly(I:C). (B) HB knockdown inhibited IFN- β activation by CSFV or SeV infection in PK-15 cells. *P* values are indicated above the bars. The data are representative of three independently conducted experiments. (C) Expression of endogenous HB protein in PK-15 cells treated with IFN- β at various times. (D) Quantitative RT-PCR analyses of IFN- β mRNA abundance in PK-15 cells transfected with p3 $\times$ Flag-HB or the empty vector (EV) and then infected with CSFV for 48 h. *P* values are indicated above the bars. The data are representative of three independent experiments.

and Myc-C proteins were used as a control for specificity. Co-IP with anti-Flag MAb showed that Myc-C protein formed a complex with 3 $\times$ Flag-HB but not with 3 $\times$ Flag (Fig. 2A). Since the above-described experiments did not exclude the possibility that C protein-HB association might be mediated indirectly through association with another cellular protein(s), a GST pulldown assay was performed with glutathione beads conjugated to GST-C or GST protein. The presence of GST-C protein, but not GST, resulted in the binding of HB (Fig. 2B), indicating that the interaction of cellular HB with the CSFV C protein is likely due to a direct physical association. To determine whether C protein interacts with cellular HB in the context of CSFV infection, virus-infected PK-15 cell lysates were immunoprecipitated with an anti-C MAb and probed for the presence of HB with anti-HB pAb. HB was readily detected in CSFV-infected PK-15 cells (Fig. 2C), indicating that HB indeed interacts with endogenous C protein in CSFV-infected PK-15 cells. To examine the colocalization of C protein with HB, HEK293T cells were cotransfected with plasmids expressing 3 $\times$ Flag-HB and Myc-C proteins and the subcellular localization of C protein and HB was examined by confocal microscopy (Fig. 2D). Both Myc-C protein and 3 $\times$ Flag-HB were distributed throughout the cytoplasm, and C protein colocalized extensively with HB. To confirm that endogenous HB colocalizes

with C protein, PK-15 cells were infected with CSFV for 48 h and analyzed by confocal microscopy. Confocal images of the cells immunostained with anti-C and anti-HB antibodies showed colocalization of HB with CSFV C protein (Fig. 2E). On the basis of digital analysis of multiple cell images, 90 and 98% of the HB-labeled pixels colocalized with exogenous and endogenous C protein, respectively. The colocalization coefficients were 0.90 and 0.98, respectively. Collectively, these findings confirm that HB is an interacting partner of CSFV C protein.

The C-terminal portion of HB is necessary for interaction with C protein. To determine which domain is necessary for the interaction with C protein, a number of mutant forms of swine HB were generated (Fig. 3A) and assessed for the capacity to interact with C protein. Notably, the C-terminal deletion mutant form of HB (Δ 100-146) lost the ability to associate with C protein (Fig. 3B).

Depletion of HB by siRNAs enhances CSFV growth and C protein expression. To investigate the relevance of the C-HB interaction to the CSFV life cycle, specific siRNAs were used to target HB in PK-15 cells, resulting in efficient knockdown of the protein (Fig. 4A). Knockdown of HB resulted in upregulation of CSFV C protein compared to cells treated with a scrambled siRNA (siScr), mock-treated cells (Mock), or untreated cells (No treat) (Fig. 4B).

FIG 8 HB interacts with RIG-I and upregulates its expression, and the C protein inhibits HB and RIG-I expression. (A) Co-IP analyses of interaction between HB and RIG-I or MDA5. IP, immunoprecipitation. (B) Immunoblotting analysis of HEK293T cells cotransfected with plasmids expressing HB (1, 2, or 4 μ g; wedges) and MDA5 (3 μ g) or RIG-I (3 μ g) (+) or empty vectors (-). (C) Quantitation of RIG-I and MDA5 protein levels with Odyssey software (Li-COR) from three independent experiments as described for panel B. *P* values are indicated above the bars. Data are representative of results of three independent experiments. (D) C protein inhibits the expression of HB and RIG-I. Immunoblotting analysis of HEK293T cells cotransfected with plasmids expressing HB (1 or 3 μ g; wedges), C protein (1 or 3 μ g; black rectangular box), and RIG-I (2 μ g) (+) or empty vectors (-).

In addition, HB-silenced cells exhibited an increased viral titer in the supernatant (Fig. 4C). These results indicate that knockdown of cellular HB enhances virus growth and C protein expression.

Overexpression of HB suppresses CSFV growth and C protein expression. The observation that depletion of HB resulted in enhanced CSFV protein expression and growth prompted an examination of the effects of HB overexpression on CSFV. PK-15 cells were transiently transfected with 3×Flag-HB and subsequently infected with CSFV. CSFV C protein was reduced when HB was upregulated (Fig. 5A). A reduction of viral titers was observed in the supernatants of these cells (Fig. 5B). Together with the results of the knockdown experiments, these findings highlight the antagonistic effect of cellular HB expression on CSFV growth and C protein expression.

HB affects viral genomic replication, and CSFV inhibits HB expression. To examine whether HB plays a role in CSFV RNA synthesis, PK-15 cells that were depleted of HB by siRNA were infected with CSFV. Examination of the genomic copies of CSFV RNA in HB knockdown cells revealed that the synthesis of viral RNA was significantly increased compared to that in control cells

(No Treat, Mock, and siScr in Fig. 6A). Conversely, overexpression of HB resulted in the downregulation of CSFV genomic replication (Fig. 6B). The results suggest that HB has a significant inhibitory effect on CSFV genomic replication. Furthermore, the expression of HB in PK-15 cells infected with CSFV was assessed and the results indicated that CSFV inhibits the expression of endogenous HB (Fig. 6C).

HB activates IFN- β production. IFN actually results in an increase in dimethyl sulfoxide-induced hemoglobin synthesis (33). To examine whether HB affects IFN production, the effect of overexpression of HB on poly(I:C)-triggered reporter gene activation was examined. HB increased the poly(I:C)-induced IFN- β transcription level in HEK293T cells (Fig. 7A), which is indicative of a positive role for HB in the cellular antiviral response. In contrast, knockdown of HB by siRNAs significantly inhibited IFN- β expression in SeV- or CSFV-infected PK-15 cells (Fig. 7B), indicating that HB positively regulates IFN- β transcription. In addition, the expression of HB in PK-15 cells pretreated with IFN- β was examined and the results showed that IFN- β increased the expression of endogenous HB (Fig. 7C). Furthermore, IFN- β mRNA was induced in either HB-overexpressing or CSFV-infected HB-

FIG 9 Endogenous swine RIG-I or IFN-β restricts CSFV growth and replication in PK-15 or SK6 cells. (A) Western blotting of endogenous swine RIG-I expression with goat anti-RIG-I pAb in PK-15 cells transfected with siRNA (siRIG-I-1, siRIG-I-2, and siRIG-I-3). Titrers (B) and numbers of genomic copies (C) of CSFV in PK-15 cells transfected with siRNA were determined. Titrers (D) and numbers of genomic copies (E) of CSFV in SK6 cells treated with IFN-β at 48 h postinfection were also determined. The data shown are representative of three independent experiments. *P* values are indicated above the bars. (F) Western blotting of CSFV C protein expression in SK6 cells treated with IFN-β at 48 h after CSFV infection. (G) Western blotting of the expression of HB and C proteins in PK-15 cells transfected with siRNA and p3×Flag-HB for 48 h after CSFV infection at various time points. Titrers (H) and numbers of genomic copies (I) of CSFV in PK-15 cells transfected with siRNA and p3×Flag-HB for 48 h after CSFV infection at various time points. The data shown are representative of three independent experiments. *P* values are indicated above the bars. EV, empty vector.

overexpressing PK-15 cells (Fig. 7D). Collectively, HB activates IFN-β production.

RIG-I interacts with and is upregulated by HB. The double-stranded RNA produced by RNA viruses during viral replication is recognized by sensor molecules such as RIG-I, MDA5, and LGP2. These sensors initiate signaling cascades including the activation of transcription factors (IFN regulatory factor 3 [IRF-3], IRF-7) and IFN genes (34). Thus, the potential for HB to interact with the positive regulators of IFN activity, RIG-I and MDA5, was investigated. HEK293T cells were cotransfected with expression plasmids encoding HB and either RIG-I or MDA5. HB was found to selectively interact with RIG-I but not with MDA5 (Fig. 8A). This finding prompted us to investigate whether HB affects the expression of MDA5 or RIG-I. Western blotting and quantitative analysis revealed that overexpression of HB resulted in the increased expression of RIG-I, but not MDA5 (Fig. 8B and C). To explore the effect of the C protein on the expression of RIG-I and HB, HEK293T cells were cotransfected with plasmids pMyc-C, pMyc-

RIG-I, and p3×Flag-HB. The expression levels of both HB and RIG-I were reduced with the increased expression of the C protein (Fig. 8D). These data suggest that HB inhibits CSFV growth via triggering of RIG-I activation and the C protein inhibits RIG-I expression.

Depletion of RIG-I by siRNAs or lack of IFN-β enhances CSFV growth, genomic replication, and C protein expression. To further address the role of endogenous RIG-I in CSFV infection, the endogenous RIG-I expression in PK-15 cells was knocked down by siRNA targeting the swine RIG-I gene (Fig. 9A). The results showed that knockdown of RIG-I increased CSFV growth and replication in PK-15 cells (Fig. 9B and C). To further ascertain whether IFN has effects on CSFV growth and replication, SK6 cells, which are deficient in type I IFN production (35), were infected with CSFV after treatment with IFN-β. The results indicated that SK6 cells were more efficiently infected with CSFV than those pretreated with IFN-β (Fig. 9D, E, and F). Further, to determine whether HB is still effective at suppressing virus infection in

the absence of RIG-I, we detected the expression of HB and C proteins and CSFV growth and replication in PK-15 cells transfected with siRNA and p3×Flag-HB after infection with CSFV (Fig. 9G, H, and I). The results showed that HB was unable to suppress CSFV infection in the absence of RIG-I in PK-15 cells. Overall, depletion of RIG-I or lack of IFN- β enhances CSFV growth, replication, and C protein expression.

DISCUSSION

Because of their limited genome coding capacity, viruses depend on host factors to carry out important functions. During the course of evolution, individual viruses have acquired a unique array of multifunctional proteins. In an attempt to identify host cellular proteins that interact with the C protein of CSFV and understand the functional importance of these interactions, GST pulldown and MS were employed to isolate host factors that interact with the CSFV C protein. Among the cellular proteins screened, the HB protein was identified and selected for further analysis. By confocal analysis, C protein and HB were found to interact (Fig. 2C) and colocalize in the cytoplasm (Fig. 2D). It is likely that the CSFV C protein translocates to the nucleus during later phases of infection, because the CSFV C protein has been shown to influence cellular transcription (5).

Hemoglobin is the red blood cell protein that carries oxygen. It was found in the red blood cells of all vertebrates, as well as the tissues of some invertebrates, such as insects, worms, clams, crabs, octopi, snails, and starfish (36). In this study, we showed that HB exists in all of the swine tissues and cell tested. In addition, we found that the abundance of HB in swine red blood cells is much higher than that in other cell types and that β -tubulin, which was used as the loading control, is hardly detectable in swine red blood cells (data not shown). Previous studies paid little attention to hemoglobin in mammalian tissues and cells other than red blood cells, possibly because of its relatively lower abundance.

It has been shown that interaction of the HCV C protein with cellular molecules impairs the host's immune response through mechanisms that result in the suppression of interleukin-12 synthesis in human macrophages (37), T cell dysfunction (38), and inhibition of T-lymphocyte activation and proliferation (25, 39, 40). In Friend leukemia cells treated with IFN, the induction and production of leukemia viruses are inhibited while induction of HB is slightly increased (33). On the basis of these findings, we speculated that the interaction between C and HB influences IFN signaling. It has been shown that viral invasion triggers an array of host innate immune antiviral responses, resulting in the production of various cytokines and chemokines. To detect viral infections, cells use pattern recognition receptors to sense viral nucleic acids (41). RIG-I-like helicases, including RIG-I and MDA5, function as cytoplasmic RNA sensors that recognize viral RNAs released during virus replication (42). We found that HB mediated IFN signaling by interacting with RIG-I, that HB-mediated suppression of CSFV growth and replication involved the upregulation of RIG-I and IFN- β , and that CSFV growth and replication were increased when RIG-I was knocked down in PK-15 cells. To examine the change of HB in PK-15 cells infected with CSFV or treated with IFN- β , we detected the expression of HB at different time points after infection with CSFV or treatment with IFN- β . We showed that CSFV/C inhibited the expression of HB, that IFN- β upregulated the expression of HB and inhibited CSFV growth and replication, and that the C protein antagonized HB-

FIG 10 Proposed relationships among CSFV or C protein, HB, RIG-I, and IFN- β . The expression of HB is inhibited when PK-15 cells are infected with CSFV or express the C protein. HB activates IFN- β via upregulation of RIG-I, while IFN- β regulates HB expression and inhibits CSFV growth.

mediated upregulation of RIG-I. We proposed a possible relationship among CSFV/C, HB, RIG-I, and IFN- β , as illustrated in Fig. 10. In short, CSFV or C protein alone inhibits the expression of HB, HB activates RIG-I, RIG-I activates IFN- β , and IFN- β inhibits CSFV infection and increases HB expression. What activates HB, how it does so, and how HB activates RIG-I signaling during viral infection need to be studied further.

The key findings of this study are the identification of cellular HB as a novel interacting partner of the CSFV C protein and the demonstration that HB inhibits the replication and growth of CSFV through activation of the IFN signaling pathway. Additional studies are needed to define the role of HB as a novel antiviral restriction factor.

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