Nonstructural Protein 4 of Porcine Reproductive and Respiratory Syndrome Virus Modulates Cell Surface Swine Leukocyte Antigen Class I Expression by Downregulating β 2-Microglobulin Transcription

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ABSTRACT Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of PRRS, which has important impacts on the pig industry. PRRSV infection results in disruption of the swine leukocyte antigen class I (SLA-I) antigen presentation pathway. In this study, highly pathogenic PRRSV (HP-PRRSV) infection inhibited transcription of the β 2-microglobulin (β 2M) gene (*B2M*) and reduced cellular levels of β 2M, which forms a heterotrimeric complex with the SLA-I heavy chain and a variable peptide and plays a critical role in SLA-I antigen presentation. HP-PRRSV nonstructural protein 4 (Nsp4) was involved in the downregulation of β 2M expression. Exogenous expression of Nsp4 downregulated β 2M expression at both the mRNA and the protein level and reduced SLA-I expression on the cell surface. Nsp4 bound to the porcine *B2M* promoter and inhibited its transcriptional activity. Domain III of Nsp4 and the enhancer *PAM* element of the porcine *B2M* promoter were identified as essential for the interaction between Nsp4 and *B2M*. These findings demonstrate a novel mechanism whereby HP-PRRSV may modulate the SLA-I antigen presentation pathway and provide new insights into the functions of HP-PRRSV Nsp4.

IMPORTANCE PRRSV modulates the host response by disrupting the SLA-I antigen presentation pathway. We show that HP-PRRSV downregulates SLA-I expression on the cell surface via transcriptional inhibition of *B2M* expression by viral Nsp4. The interaction between domain III of Nsp4 and the enhancer *PAM* element of the porcine *B2M* promoter is essential for inhibiting *B2M* transcription. These observations reveal a novel mechanism whereby HP-PRRSV may modulate SLA-I antigen presentation and provide new insights into the functions of viral Nsp4.

KEYWORDS β 2-microglobulin, Nsp4, porcine reproductive and respiratory syndrome virus, PRRSV, SLA-I, porcine *B2M* promoter

Major histocompatibility complex class I (MHC-I) antigens in pigs are termed swine leukocyte antigen class I (SLA-I), which comprises a heterotrimeric complex consisting of the SLA-I heavy chain (SLA-I HC), β 2-microglobulin (β 2M), and a variable peptide. In pigs, SLA-I is expressed on the surfaces of all nucleated cells to display peptide fragments derived from invading viruses for T cell recognition. This antigen presentation pathway is essential for the immune system to recognize viral peptides and initiate appropriate immune responses against invading viruses (1, 2).

Received 30 August 2016 Accepted 8 December 2016

Accepted manuscript posted online 21 December 2016

Citation Qi P, Liu K, Wei J, Li Y, Li B, Shao D, Wu Z, Shi Y, Tong G, Qiu Y, Ma Z. 2017. Nonstructural protein 4 of porcine reproductive and respiratory syndrome virus modulates cell surface swine leukocyte antigen class I expression by downregulating β 2-microglobulin transcription. *J Virol* 91:e01755-16. <https://doi.org/10.1128/JVI.01755-16>.

Editor Julie K. Pfeiffer, University of Texas Southwestern Medical Center

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The SLA-I antigen presentation pathway is tightly regulated. Typically, the SLA-I HC and β 2M form a heterodimeric complex in the endoplasmic reticulum (ER). A viral peptide generated by proteasomal degradation within the virus-infected cells then binds to a groove in the SLA-I HC- β 2M complex to form a heterotrimeric SLA-I complex, which is subsequently expressed on the cell surface, where it becomes available for immune surveillance by T cells (3). Because of its critical role in initiating an immune response to virus infection, the SLA-I antigen presentation pathway is targeted by viruses at all essential stages. For example, viruses may inhibit SLA-I synthesis, block the peptide-binding groove, target SLA-I for proteasomal and lysosomal degradation, or retain SLA-I in the ER (2). Targeting of the SLA-I antigen presentation pathway by viruses leads to impaired expression of SLA-I on the cell surface and subsequent suppression of the host antiviral immune response.

β 2M is an approximately 13-kDa protein encoded by the *B2M* gene, which associates with the SLA-I HC and plays an essential role in SLA-I cell surface expression. β 2M-deficient mice showed limited SLA-I expression on the cell surface, no mature CD4⁺CD8⁺ T cells, and defective CD4⁺CD8⁺ T cell-mediated cytotoxicity (4). Its critical role in the SLA-I antigen presentation pathway means that β 2M is also targeted by invading pathogens. For example, the UL18 protein of human cytomegalovirus binds to β 2M and affects SLA-I expression on the cell surface (5), while ESAT-6 protein of *Mycobacterium tuberculosis* sequesters β 2M in the ER to impair SLA-I expression on the cell surface, resulting in downregulation of the SLA-I antigen presentation pathway (6).

Porcine reproductive and respiratory syndrome virus (PRRSV), also called classical PRRSV, is the causative agent of PRRS and first emerged in the United States in 1987 (7). A highly pathogenic PRRSV (HP-PRRSV) strain characterized by high and continuous fever and high morbidity and mortality emerged in China in 2006 (8). The genetic difference between HP-PRRSV and classical PRRSV strains is a unique discontinuous deletion of 30 amino acids in nonstructural protein 2 (Nsp2) of HP-PRRSV, which is a genetic marker of the HP-PRRSV strain differentiating it from the classical PRRSV strain. A PRRSV strain with the unique discontinuous deletion of 30 amino acids in Nsp2 is considered an HP-PRRSV strain (8, 9). Both classical PRRSV and HP-PRRSV strains circulate in pig herds and have important impacts on the pig industry. PRRSV (family *Arteriviridae*, order *Nidovirales*) is an enveloped RNA virus with a positive-sense single-stranded RNA genome that encodes eight structural proteins (GP2a, E, GP3, GP4, GP5, GP5a, M, and N) and at least 16 Nsps (Nsp1 α , Nsp1 β , Nsp2 to -6, Nsp2TF, Nsp2N, Nsp7 α , Nsp7 β , and Nsp8 to -12) (10). PRRSV infection results in immune suppression, including delayed, low-level induction of cytotoxic T lymphocyte responses in pigs (11), which is thought to relate to disruption of the SLA-I antigen presentation pathway (12). PRRSV infection has been shown to downregulate SLA-I expression on the cell surface (13, 14). Proteasomal degradation of SLA-I HC by HP-PRRSV Nsp1 α is responsible for the downregulation of SLA-I expression on the cell surface (12). In this study, we investigated the mechanisms whereby HP-PRRSV impairs SLA-I expression and show that HP-PRRSV Nsp4 binds to the porcine *B2M* promoter to inhibit β 2M transcription, thereby impairing SLA-I expression on the cell surface.

RESULTS

HP-PRRSV reduces SLA-I expression on the cell surface. HP-PRRSV has a stronger inhibitory effect on the host antiviral immune response than classical PRRSV (15). We therefore used a strain of HP-PRRSV in the current study. Although different PRRSV strains, including HP-PRRSV and classical PRRSV strains, have been shown to downregulate SLA-I expression on the cell surface (11–14), the inhibitory effects on host antiviral immune responses vary among different PRRSV strains (15), and we therefore confirmed that the HP-PRRSV strain used in this study also downregulated SLA-I expression on the cell surface. We infected porcine alveolar macrophages (PAMs), which are the major target cells of PRRSV infection (16), with HP-PRRSV at multiplicities of infection (MOI) of 2 and 5, harvested them at 24 h postinfection (hpi), and detected SLA-I expression on the cell surface by flow cytometry using antibody against the SLA-I

FIG 1 Downregulation of SLA-I expression on the cell surface by HP-PRRSV infection. PAMs (A) and Marc-145 cells (B) were mock infected (Mock) or infected with HP-PRRSV at MOI of 2 and 5 and incubated for 24 h. The expression levels of SLA-I on the cell surface were measured by flow cytometry with anti-SLA-I HC monoclonal antibodies and are presented as mean fluorescence intensities (MFI). Data are presented as means $\pm$ SD from three independent experiments. *, $P < 0.05$ between HP-PRRSV- and mock-infected cells as determined by Student's *t* test.

HC. Following HP-PRRSV infection, SLA-I expression on the surfaces of PRRSV-infected PAMs was significantly reduced compared with that of mock-infected PAMs (Fig. 1A). HP-PRRSV also inhibited SLA-I expression on the cell surfaces of Marc-145 cells, which are susceptible to PRRSV infection (17) (Fig. 1B). These observations indicate that the HP-PRRSV strain used in this study was able to downregulate SLA-I expression on the cell surface, in agreement with previous observations (11–14).

Proteasomal degradation is partially responsible for downregulation of cellular β 2M protein levels. Both β 2M and SLA-I HC are targeted by viruses to modulate SLA-I expression on the cell surface (3, 5). We explored the molecular basis of HP-PRRSV-induced downregulation of SLA-I expression on the cell surface by determining changes in the cellular levels of β 2M and SLA-I HC proteins by Western blotting in Marc-145 cells in response to HP-PRRSV infection. HP-PRRSV replication was indicated by detection of viral N protein. Both β 2M and SLA-I HC expression levels were significantly reduced in HP-PRRSV-infected cells compared with those in mock-infected cells (Fig. 2A and B), demonstrating that HP-PRRSV infection reduced the cellular abundance of β 2M and SLA-I HC proteins.

Targeting of the SLA-I complex for proteasomal degradation is a common strategy employed by viruses to modulate the SLA-I antigen presentation pathway (3). We determined if proteasomal degradation contributed to the downregulation of cellular β 2M and SLA-I HC proteins in HP-PRRSV-infected cells by treating HP-PRRSV- and mock-infected Marc-145 cells with PYR-41 (18) to block downstream ubiquitination and ubiquitination-dependent proteasomal degradation (Fig. 2C), followed by Western blot analysis. HP-PRRSV infection reduced the cellular levels of β 2M and SLA-I HC proteins at 24 h with dimethyl sulfoxide (DMSO) (Fig. 2D), consistent with the results in Fig. 2A. PYR-41 treatment blocked the downregulation of SLA-I HC by HP-PRRSV and restored it to mock-infection levels (Fig. 2D), suggesting that proteasomal degradation was responsible for SLA-I HC downregulation, in accordance with previous observations (11). However, although β 2M protein levels in HP-PRRSV-infected cells treated with

FIG 2 Cellular levels of β 2M protein in HP-PRRSV-infected cells. Marc-145 cells were mock infected (Mock) or infected with HP-PRRSV at an MOI of 2 and incubated at 37°C for the indicated times. Cellular levels of β 2M, the SLA-I HC, GAPDH, and PRRSV N were detected by Western blotting. (A) Infected Marc-145 cells were harvested at 24 and 30 h postinfection (hpi) and subjected to Western blot analysis. (B) Intensities of protein bands were determined by densitometric analysis. Relative cellular levels of β 2M and the SLA-I HC were normalized to GAPDH levels and are presented relative to the level in mock-infected cells at 24 hpi (set as 1). (C) Schematic representation of PYR-41 treatment. Infected Marc-145 cells were treated with PYR-41 at 125 μ M or mock treated with DMSO and incubated at 37°C for the indicated times. (D) Cellular levels of the indicated proteins in PYR-41-treated cells were determined by Western blotting. (E) Intensities of protein bands were determined by densitometric analysis. Relative cellular levels of β 2M were normalized to GAPDH levels and are presented relative to the levels in mock-infected and DMSO-treated cells (set as 1). Data are presented as means $\pm$ SD from three independent experiments. **, $P < 0.01$ between the indicated groups analyzed by Student's t test.

PYR-41 for 12, 18, and 24 h were higher than in HP-PRRSV-infected cells treated with DMSO for 24 h (Fig. 2D), levels were still significantly lower than in mock-infected cells treated with PYR-41 (Fig. 2D and E). These results indicate that proteasomal degradation is only partially responsible for the downregulation of β 2M by PRRSV and that additional mechanisms may be involved in this process.

HP-PRRSV downregulates β 2M expression at the transcriptional level. We explored the additional mechanisms responsible for the downregulation of β 2M by analyzing the effect of HP-PRRSV infection on β 2M expression at the transcriptional level by quantitative real-time reverse transcription-PCR (qRT-PCR). The mRNA abundance of PRRSV N protein was probed to indicate PRRSV replication. mRNA levels of *SLA-I*, encoding the SLA-I HC, were similar in HP-PRRSV- and mock-infected PAMs (Fig. 3A), suggesting that HP-PRRSV infection had no effect on the transcription of the *SLA-I* gene. However, *B2M* mRNA levels were significantly reduced in HP-PRRSV-infected PAMs compared with levels in mock-infected PAMs (Fig. 3A). *B2M* gene expression was also reduced in Marc-145 cells infected with HP-PRRSV (Fig. 3B). We also determined the dynamics of *B2M* transcription in Marc-145 cells during HP-PRRSV infection. *B2M* gene expression was significantly reduced at 18, 24, and 30 hpi with HP-PRRSV compared with that in mock-infected cells (Fig. 3C). These results indicate that HP-PRRSV infection inhibited the transcription of the *B2M* gene. To the best of our knowledge, this is the

FIG 3 Downregulation of β 2M transcription by HP-PRRSV infection. PAMs (A) and Marc-145 cells (B) were mock infected (Mock) or infected with HP-PRRSV at an MOI of 2 and incubated at 37°C for 24 h. mRNA levels of *B2M*, *SLA-I*, and PRRSV N were determined by qRT-PCR. Relative mRNA levels of each gene were normalized to levels of the GAPDH housekeeping gene and plotted. (C) Marc-145 cells were mock infected (Mock) or infected with HP-PRRSV at an MOI of 2 and incubated at 37°C for the indicated times. mRNA levels of *B2M* were determined by qRT-PCR. Relative mRNA levels of *B2M* were normalized to GAPDH levels and are expressed relative to the level in mock-infected cells at 6 h postinfection (hpi) (set as 1). Data are presented as means $\pm$ SD from three independent experiments. **, $P < 0.01$ between the indicated groups analyzed by Student's t test.

first evidence of the downregulation of β 2M expression by HP-PRRSV at the transcriptional level.

Nsp4 reduces cellular levels of β 2M protein and impairs SLA-I expression on the cell surface. Given that the Tat protein of human immunodeficiency virus downregulates β 2M expression (19), we screened for HP-PRRSV proteins responsible for downregulating β 2M expression. Five Flag-tagged HP-PRRSV proteins (Flag-GP5, Flag-N, Flag-Nsp2, Flag-Nsp4, and Flag-Nsp5) were transiently expressed in Marc-145 cells, and their effects on β 2M and SLA-I HC protein levels were determined by Western blotting. None of the expressed viral proteins had any significant effect on the cellular abundance of SLA-I HC protein, but Flag-Nsp2 and Flag-Nsp4 significantly reduced the cellular abundance of β 2M protein (Fig. 4A and B), with Flag-Nsp4 having a greater effect than Flag-Nsp2. Furthermore, Nsp4 is a 3C-like serine protease responsible for most nonstructural protein processing and for antagonizing interferon (IFN) expression (20, 21), playing an essential role in PRRSV infection, and we therefore selected Nsp4 for further studies.

Nsp4 reduced β 2M protein levels, and we therefore determined whether it inhibited SLA-I expression on the cell surface. We transiently expressed Flag-Nsp4 in PK-15 cells (a cell line used to analyze the effect of PRRSV on SLA-I expression [11, 12]) and determined its effect on SLA-I expression on the cell surface by flow cytometry. Flag-N was expressed as a negative control. Flag-Nsp4 significantly reduced SLA-I expression on the cell surface compared with Flag-N and the Flag-labeled vector (Flag-vector) (Fig. 4C and D). Flag-Nsp4 also significantly reduced SLA-I expression on the surfaces of Marc-145 cells (Fig. 4E). These results indicate that PRRSV Nsp4 was responsible for reducing the cellular abundance of β 2M protein and impairing SLA-I expression on the cell surface.

Nsp4 binds to the porcine *B2M* promoter and represses its transcription. We explored the mechanisms whereby Nsp4 reduced β 2M expression by examining the effect of exogenous Flag-Nsp4 expression on *B2M* transcription in Marc-145 and PK-15 cells. As expected, the abundance of *B2M* mRNA was significantly reduced in cells expressing Flag-Nsp4 compared with that in cells expressing Flag-N or the Flag-vector (Fig. 5A and B). Because of a lack of information on the porcine *B2M* promoter, we cloned the upstream region of the porcine *B2M* gene based on that of the swine

FIG 4 Nsp4 reduces the cellular level of $\beta 2M$ protein. (A) Marc-145 cells were transfected with the indicated plasmids and incubated at 37°C for 24 h. The expression of Flag-tagged viral proteins in the transfectants was probed by Western blotting with anti-Flag antibody. Cellular levels of $\beta 2M$, the SLA-I HC, and GAPDH were detected by Western blotting. (B) Intensities of protein bands were determined by densitometric analysis. Relative cellular levels of $\beta 2M$ in the transfectants were normalized to GAPDH levels and are presented relative to the level in cells expressing Flag-N (set as 1). PK-15 (C and D) and Marc-145 (E) cells were transfected with plasmids expressing Flag-Nsp4, Flag-N, or the Flag-vector and incubated at 37°C for 24 h. Expression levels of SLA-I on the cell surface were measured by flow cytometry with anti-SLA-I monoclonal antibodies and are presented as mean fluorescence intensities (MFI). Data are presented as means $\pm$ SD from three independent experiments. **, $P < 0.01$ between the indicated groups analyzed by Student's t test.

reference genome (*Sus scrofa* Sscrofa10.2) and derived the porcine *B2M* promoter region from the murine *B2M* promoter region containing the basal promoter sequence elements and the upstream enhancers, including *PAM*, and the IFN consensus sequence (*ICS*) (22) (Fig. 5C). The cloned porcine *B2M* promoter from nucleotides -1817 to $+59$ was inserted into a pGL3 luciferase reporter vector to generate the luciferase reporter plasmid (-1817 B2M) (Fig. 5C). The inhibitory effect of Nsp4 on the porcine *B2M* promoter was determined by coexpression of Flag-Nsp4 and the luciferase reporter plasmid -1817 B2M in PK-15 cells. Expression of Flag-N had no significant effect on luciferase activity, but expression of Flag-Nsp4 significantly reduced the luciferase activity of -1817 B2M compared with the level resulting after expression of Flag-N or the Flag-vector (Fig. 5D), suggesting that Nsp4 represses porcine *B2M* promoter activity. Binding of a viral protein to a host gene promoter is a mechanism employed by viruses to repress transcription (23). We determined whether Nsp4 bound to the porcine *B2M* promoter by a chromatin immunoprecipitation (ChIP) assay using PK-15 cells transfected with Flag-Nsp4. Anti-Flag antibodies immunoprecipitated a DNA fragment containing part of the porcine *B2M* promoter ranging from nucleotides -218 to -181 , as confirmed by DNA sequencing (data not shown), in cells expressing Flag-Nsp4 but not in cells expressing Flag-N (Fig. 5E). Nsp4 is known to localize in the nucleolus to inhibit IFN- β transcription (24), and we therefore detected the subcellular localization of Flag-Nsp4 expressed in PK-15 cells by immunofluorescence assay. Flag-Nsp4 was distributed mainly in the nucleolus, as demonstrated by its colocalization with the nuclear marker lamins (Fig. 5F). These results indicate that Nsp4 binds to the porcine *B2M* promoter and represses its transcription.

Domain III of Nsp4 downregulates $\beta 2M$ protein and represses *B2M* promoter activity. Nsp4 is folded into three domains (20) (Fig. 6A). We generated three plasmids

FIG 5 Nsp4 inhibits transcription of *B2M*. Marc-145 (A) and PK-15 (B) cells were transfected with plasmids expressing Flag-Nsp4, Flag-N, or the Flag-vector and incubated at 37°C for 24 h. Levels of *B2M* mRNA in the transfectants were determined by qRT-PCR. Relative levels of *B2M* mRNA were normalized to levels of the GAPDH housekeeping gene and are expressed relative to the level in cells transfected with the Flag-vector (set as 1). (C) Schematic representation of the porcine *B2M* promoter region and luciferase reporter plasmids. (D) PK-15 cells were cotransfected with the luciferase reporter plasmid – 1817 B2M and a plasmid expressing Flag-Nsp4, Flag-N, or the Flag-vector. Luciferase activities of the transfectants were analyzed at 24 h posttransfection. Luciferase activity was normalized to *Renilla* luciferase activity and is presented relative to the activity in cells transfected with the Flag-vector (set as 1). (E) PK-15 cells were transfected with a plasmid expressing Flag-Nsp4 or Flag-N and incubated at 37°C for 24 h. Transfectants were harvested 24 h posttransfection and subjected to ChIP assay with anti-Flag antibody. The bound DNA fractions were detected by PCR with appropriate primers (Table 1). PCR products were analyzed by 4% agarose gel electrophoresis. (F) PK-15 cells were transfected with a plasmid expressing Flag-Nsp4 or Flag-Nsp4 domains and incubated at 37°C for 24 h. The subcellular localization of the expressed Nsp4 proteins (green) was visualized by immunofluorescence. The subcellular localization of the expressed Nsp4 proteins (green) was visualized by immunofluorescence. Nuclear marker lamins (red) were probed with anti-lamin A/C antibody to indicate the nuclei. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Data are presented as means $\pm$ SD from three independent experiments. ***P* < 0.01 between the indicated groups analyzed by Student's *t* test.

expressing Flag-tagged mutant domains of Nsp4 to map the domain responsible for inhibiting *B2M* expression (Fig. 6A). We transfected Marc-145 cells with these plasmids and analyzed the cellular abundance of β 2M in the transfectants by Western blotting. Flag-Nsp4 expression was associated with a significant decrease in β 2M abundance compared with that produced with Flag-N expression (Fig. 6B and C), consistent with the results shown in Fig. 4A. β 2M expression was significantly inhibited by expression of Flag-Nsp4-domain III, but not Flag-Nsp4-domain I or Flag-Nsp4-domain II (Fig. 6B and C), indicating that domain III of Nsp4 was responsible for the downregulation of β 2M. We investigated the effect of Flag-Nsp4-domain III on porcine *B2M* promoter activity

FIG 6 Domain III of Nsp4 inhibits β 2M expression. (A) Schematic representation of Nsp4 domains and truncated mutant Nsp4 domains. (B) Marc-145 cells were transfected with the indicated plasmids and incubated at 37°C for 24 h. Expression levels of β 2M, GAPDH, and Flag-tagged viral proteins were determined by Western blotting. (C) Intensities of protein bands were determined by densitometric analysis. Relative cellular levels of β 2M in the transfectants were normalized to GAPDH levels and are presented relative to the level in cells expressing Flag-N (set as 1). (D) PK-15 cells were cotransfected with the luciferase reporter plasmid -1817 B2M and the plasmid expressing Flag-Nsp4, the Flag-Nsp4 domains, Flag-N, or the Flag-vector. Luciferase activities of the transfectants were analyzed at 24 h posttransfection. Luciferase activity was normalized to *Renilla* luciferase activity and is presented relative to the activity in cells transfected with the Flag-vector (set as 1). Data are presented as means $\pm$ SD from three independent experiments. **, $P < 0.01$ between the indicated groups analyzed by Student's t test.

further and found that Flag-Nsp4-domain III, but not Flag-Nsp4-domain I or Flag-Nsp4-domain II, significantly reduced luciferase activities (Fig. 6D), suggesting that domain III of Nsp4 repressed *B2M* transcription. Nsp4 was localized mainly in the nucleolus, as shown in Fig. 5F. We determined whether domain III of Nsp4 was localized in the nucleolus by immunofluorescence assay. In contrast to Flag-Nsp4-domain I and Flag-Nsp4-domain II, Flag-Nsp4-domain III was distributed in the nucleolus (Fig. 5F). These observations indicate that Nsp4 domain III is responsible for the downregulation of β 2M abundance and *B2M* promoter activity.

Domain III of Nsp4 binds to the PAM element of the *B2M* promoter to repress *B2M* transcriptional activity. We mapped the region of the porcine *B2M* promoter targeted by Nsp4 by inserting a series of truncated *B2M* promoters into a pGL3 luciferase reporter vector to generate luciferase reporter plasmids (Fig. 5C). The transcriptional activity of each truncated *B2M* promoter was determined in PK-15 cells by luciferase assay. Truncation of the *B2M* promoter resulted in reduced luciferase activities (Fig. 7A), suggesting essential roles for the enhancer elements, including PAM and ICS (Fig. 5C). We determined the inhibitory effect of Nsp4 on the transcriptional activity of each truncated *B2M* promoter by coexpression of the luciferase reporter plasmids with Flag-Nsp4 in PK-15 cells, followed by determination of the luciferase activities. Cotransfection of luciferase reporter plasmids with Flag-N or the Flag-vector was used as a control. Flag-Nsp4 had no significant effect on the luciferase activities of the -163 B2M and -181 B2M truncation *B2M* promoters but significantly reduced the luciferase activities of the -218 B2M, -318 B2M, and -1817 B2M truncation promoters (Fig. 7B). This implies that the Nsp4-targeted region might be located between nucleotides -181 and -218, in accordance with the result of the ChIP assay (Fig. 5E). Analysis of the sequence between nucleotides -181 and -218 showed a putative PAM element (ATCATA) within this region (Fig. 5C). The PAM element is an enhancer identified in the murine *B2M* promoter (22) and targeted by the Tat protein of human immunodeficiency virus to repress *B2M* expression (19). We therefore determined whether the PAM element was a target of Nsp4. We deleted the PAM element from the porcine *B2M*

FIG 7 The PAM element is necessary for Nsp4-domain III binding to B2M promoter. (A) PK-15 cells were transfected with the indicated luciferase reporter plasmids containing a series of truncated B2M promoters. The transcriptional activity of each truncated B2M promoter was determined at 24 h posttransfection using luciferase assay and normalized to Renilla luciferase activity. (B) PK-15 cells were cotransfected with the indicated luciferase reporter plasmids and a plasmid expressing Flag-Nsp4, Flag-N, or the Flag-vector. The transcriptional activity of each truncated B2M promoter was determined at 24 h posttransfection using a luciferase assay. The luciferase activity of each transfectant was normalized to Renilla luciferase activity and is presented relative to the activity in cells cotransfected with each truncated B2M promoter and the Flag-vector (set as 1). (C) PK-15 cells were cotransfected with a luciferase reporter plasmid containing -1817 B2M or -1817 Δ PAM B2M and a plasmid expressing Flag-Nsp4, Flag-Nsp4-domain III, Flag-N, or the Flag-vector. The transcriptional activity of each truncated B2M promoter was determined at 24 h posttransfection by a luciferase assay. The luciferase activity of each transfectant was normalized to Renilla luciferase activity and is presented relative to the activity in cells cotransfected with each truncated B2M promoter and the Flag-vector (set as 1). (D) Vero cells were cotransfected with a luciferase reporter plasmid containing -1817 B2M or -1817 Δ PAM B2M and a plasmid expressing Flag-Nsp4, Flag-Nsp4-domain III, or Flag-N. The transfectants were harvested 24 h posttransfection and subjected to a ChIP assay with anti-Flag antibody. The bound DNA fractions were detected by PCR with appropriate primers (Table 1). The PCR products were analyzed by 4% agarose gel electrophoresis. Data are presented as means $\pm$ SD from three independent experiments. **, $P < 0.01$ between the indicated groups analyzed by Student's t test.

promoter and determined the promoter activity of this deletion mutant (-1817 Δ PAM B2M) (Fig. 5C) using the luciferase assay. Deletion of the PAM element resulted in a significant decrease in B2M promoter activity (Fig. 7A), suggesting that this element was required for porcine B2M promoter activity. We compared the inhibitory effect of Flag-Nsp4 on the transcriptional activities of the truncated B2M promoter -1817 B2M and the deletion mutant -1817 Δ PAM B2M. Flag-Nsp4 significantly reduced the promoter activity of -1817 B2M but showed no significant inhibitory effect on the promoter activity of -1817 Δ PAM B2M (Fig. 7C). Given that Nsp4 domain III inhibited B2M promoter activity (Fig. 6D), we also compared the inhibitory effects of Flag-Nsp4-domain III on the transcriptional activities of the truncated B2M promoter -1817 B2M and the deletion mutant -1817 Δ PAM B2M. As expected, Flag-Nsp4-domain III did not significantly inhibit the promoter activity of -1817 Δ PAM B2M (Fig. 7C). These results indicate that the PAM element is the target of Nsp4. To determine whether Nsp4-domain III bound to the PAM element, we cotransfected the truncated B2M promoter

–1817 B2M or the deletion mutant –1817 Δ PAM B2M into Vero cells with Flag-Nsp4, Flag-Nsp4-domain III, or Flag-N for ChIP assays. Both Flag-Nsp4 and Flag-Nsp4-domain III immunoprecipitated a DNA fragment that contained part of the porcine *B2M* promoter in cells transfected with the truncated *B2M* promoter –1817 B2M. However, no DNA fragment was immunoprecipitated by Flag-Nsp4 or Flag-Nsp4-domain III in cells transfected with the deletion mutant –1817 Δ PAM B2M (Fig. 7D). These data suggest that the *PAM* element is essential for Nsp4-domain III binding to the *B2M* promoter and therefore for repressing *B2M* promoter activity.

DISCUSSION

Disruption of the SLA-I antigen presentation pathway is thought to be employed by PRRSV as a strategy for modulating the host immune response (11–14), although the molecular basis of this process is unclear. PRRSV was shown previously to downregulate SLA-I expression on the cell surface via viral Nsp1 α -mediated proteasomal degradation of the SLA-I HC (11). In this study, we found that HP-PRRSV downregulated SLA-I expression on the cell surface via transcriptional inhibition of *B2M* expression by viral Nsp4, representing a novel mechanism employed by HP-PRRSV to modulate the SLA-I antigen presentation pathway.

We confirmed that the HP-PRRSV strain used in this study was able to downregulate SLA-I expression on the cell surface and showed that cellular levels of β 2M and SLA-I HC proteins were significantly decreased in response to HP-PRRSV infection. Proteasomal degradation was responsible for the downregulation of the SLA-I HC in HP-PRRSV-infected cells. These observations were consistent with those of previous studies (11). However, proteasomal degradation was only partially responsible for the downregulation of β 2M, given that inhibition of the ubiquitin-proteasome pathway by the chemical inhibitor PYR-41 (18) did not restore β 2M to mock-infection levels. We further showed that HP-PRRSV infection inhibited transcription of the *B2M* gene, encoding β 2M, by targeting the enhancer *PAM* element of the porcine *B2M* promoter. To the best of our knowledge, this is the first evidence for targeting of the porcine *B2M* promoter by PRRSV and thereby of its downregulation of *B2M* transcription as well as β 2M protein levels to modulate the SLA-I antigen presentation pathway.

A previous study described by Du et al. (11) showed that proteasomal degradation was responsible for the downregulation of β 2M during HP-PRRSV infection. However, we observed that the proteasomal degradation was only partially responsible for the downregulation of β 2M. This apparent discrepancy may be attributable to differences in the durations of chemical inhibitor treatment between our and Du et al.'s experiments. Du et al. treated virus-infected cells with the inhibitor MG132 for 8 h and observed a restoration of β 2M to mock-infection levels (11). We also observed the restoration of β 2M in virus-infected cells to mock-infection levels at 6 h posttreatment (hpt), but not at 12, 18, and 24 hpt. In addition, inhibition of the ubiquitin-proteasome pathway results in an accumulation of nonubiquitinated protein levels (25). However, no significant accumulation of the nonubiquitinated β 2M level was observed in MG132-treated cells in Du et al.'s study (11), implying that the ubiquitin-proteasome pathway might not be completely blocked by MG132 treatment at this time point. We observed a significant accumulation of the nonubiquitinated β 2M level at 12, 18, and 24 hpt but not at 6 hpt in PYR-41-treated cells. These data suggested that the treatment duration of ubiquitin-proteasome pathway inhibitors might be crucial for analysis of the proteasomal degradation of β 2M during HP-PRRSV infection.

We identified that PRRSV Nsp4 was involved in the inhibition of *B2M* transcription. Exogenous expression of Nsp4 downregulated β 2M expression at both the mRNA and protein levels, thereby impairing SLA-I expression on the cell surface. Nsp4 bound to the porcine *B2M* promoter to interfere with its transcription, and the interaction between Nsp4 domain III and the enhancer *PAM* element of the porcine *B2M* promoter was identified as essential for this inhibitory effect. Nsp4 is a 3C-like serine protease responsible for most nonstructural protein processing of PRRSV and plays an essential role in PRRSV infection (20). Nsp4 also impairs type I IFN expression by targeting the

NF- κ B essential modulator (NEMO) and virus-induced signaling adaptor (VISA) (10, 21), supporting Nsp4's critical role in modulating the host antiviral response. In this study, Nsp4 inhibited *B2M* transcription and impaired SLA-I expression on the cell surface, indicating its role in interfering with the SLA-I antigen presentation pathway and providing new insights into the functions of HP-PRRSV Nsp4.

Nsp4 consists of three domains located at amino acids 12 to 69, 89 to 153, and 157 to 199. The active site of the 3C-like serine protease of Nsp4 is located between domains I and II and harbors a canonical catalytic triad comprising Ser118, His39, and Asp64 (20). In the current study, we observed that domain III of Nsp4 bound to the enhancer *PAM* element of the porcine *B2M* promoter and contributed to inhibition of *B2M* transcription, suggesting that its 3C-like serine protease activity was not involved in this inhibitory activity. The function of Nsp4 domain III is currently unknown. Analysis of the crystal structure of the Nsp4 crystal indicates the presence of two patches of solvent-exposed hydrophobic residues in domain III, implying possible interactions with other molecules (20), thus reinforcing our observation of the binding of Nsp4 domain III to the *B2M* promoter.

A virus evolves multiple strategies to target the SLA-I antigen presentation pathway because of its critical role in initiating the host antiviral immune response (2). For example, mouse cytomegalovirus utilizes viral gp34, gp40, and gp48 to block SLA-I at the cell surface, cause its retention in the ER, and target SLA-I to lysosomes, respectively (26–28). We therefore speculated that multiple PRRSV proteins might be involved in its modulation of the SLA-I antigen presentation pathway, and Nsp1 α , Nsp2TF, and GP3 have been shown to downregulate SLA-I expression on the cell surface (11, 12). In the current study, we identified Nsp4 as being involved in the downregulation of SLA-I expression on the cell surface, though Cao et al. (12) previously reported that Nsp4 had no significant effect on SLA-I expression on the cell surface. Rapid molecular evolution is known to result in diverse PRRSV isolates with multifarious pathogenicities (29), and the apparent discrepancy between our and Cao et al.'s results may be attributable to differences in the roles of Nsp4 between the PRRSV strains used. The PRRSV strain used in this study was a highly pathogenic strain, whereas the PRRSV VR2385 strain used in Cao et al.'s study (12) was a classical PRRSV strain isolated in 1994 (30).

Previous studies showed distinct effects of Nsp4 from different pathogenic PRRSV strains on the inhibition of IFN- β induction. Nsp4 in classical PRRSV has a less inhibitory effect on the promoter activities of IFN- β , IFN regulatory factors, and NF- κ B than Nsp4 in HP-PRRSV (10, 21). Furthermore, compared with Nsp4 in HP-PRRSV, Nsp4 in classical PRRSV was unable to mediate the cleavage of VISA, a unique adaptor molecule essential for retinoic acid-induced gene 1 and melanoma differentiation-associated gene 5 signal transduction (10). These observations, together with our results, suggest that HP-PRRSV Nsp4 might play broader roles in modulating the host antiviral immune response than Nsp4 in classical PRRSV.

In conclusion, our results show that HP-PRRSV Nsp4 is involved in the downregulation of β 2M expression, thereby impairing SLA-I expression on the cell surface. Nsp4 binds to the porcine *B2M* promoter to inhibit its transcription via interactions between domain III of Nsp4 and the enhancer *PAM* element of the porcine *B2M* promoter. These findings reveal a novel mechanism employed by HP-PRRSV to modulate the SLA-I antigen presentation pathway and provide new insights into the functions of HP-PRRSV Nsp4.

MATERIALS AND METHODS

Cells and virus infection. Porcine alveolar macrophages (PAMs) were isolated from 5-week-old clinically healthy crossbred pigs raised in the animal facility of Shanghai Academy of Agricultural Sciences, Shanghai, China. The pigs tested negative for PRRSV antibody using a reproductive and respiratory syndrome virus antibody test kit (HerdChek PRRS 2XR; IDEXX Laboratories, Westbrook, ME, USA), as well as for PRRSV, porcine circovirus type 2, classical swine fever virus, and swine influenza virus by real-time PCR. Isolation of PAMs was performed as described previously (31). All animal experiments were performed in compliance with the Guidelines on the Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China, policy no. 2006 398) and were approved by the Institutional Animal Care and Use Committee at the Shanghai Veterinary Research Institute (IACUC no. Shvri-Pi-0124).

PAMs isolated from at least five pigs were pooled and cultured in RPMI 1640 medium (Thermo Fisher Scientific, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C in an atmosphere containing 5% CO₂. Marc-145, PK-15, and Vero cell lines were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C in an atmosphere containing 5% CO₂. The HP-PRRSV strain SH-PRRS01 isolated in our laboratory was used in this study. For HP-PRRSV infection, precultured cells were inoculated with HP-PRRSV at the multiplicities of infection (MOI) indicated in the figures and incubated at 37°C for 1 to 2 h. After inoculation, the inocula were removed and the cells were cultured in media containing 5% FBS for PAMs or 2% FBS for Marc-145 cells at 37°C for the indicated times. Mock-infected cells were generated using culture medium as the control inoculum.

Flow cytometry. Flow cytometry was performed as described previously (11) to determine SLA-I expression on the cell surface. Briefly, cells precultured on six-well plates were infected with HP-PRRSV at MOI of 2 or 5 and incubated at 37°C for 24 h. The infected cells were washed gently three times with cold phosphate-buffered saline (PBS), dissociated from the plates with 0.1% EDTA, and rewashed with cold PBS. The washed cells were stained with anti-SLA-I monoclonal antibody (1:100) (Kingfisher Biotech, Saint Paul, MN, USA) for PAMs or anti-MHC-I monoclonal antibody (1:100) (W6/32; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for Marc-145 cells, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (1:400) (Thermo Fisher Scientific). The fluorescence intensity of the cells was assessed, and the expression level of SLA-I on the cell surface is presented as the mean fluorescence intensity.

Drug administration. Marc-145 cells precultured on six-well plates were inoculated with HP-PRRSV at an MOI of 2 and incubated at 37°C for 24 h. Ubiquitination and ubiquitination-dependent proteasomal degradation was blocked using the chemical inhibitor of ubiquitin-activating enzyme E1, PYR-41 (Selleck Chemicals, Houston, TX, USA). PYR-41 was dissolved in culture-grade dimethyl sulfoxide (DMSO) at a concentration of 100 mM and then added at a final concentration of 125 μ M into the culture media at 6, 12, 18, and 24 h before cells were harvested for Western blot analysis. DMSO was added into the media at 24 h before harvest as a control.

Plasmids and transfection. Plasmids engineered to express Flag-tagged viral proteins, including Flag-GP5, Flag-N, Flag-Nsp2, Flag-Nsp5, Flag-Nsp4, and the Flag-Nsp4 domains, were constructed by inserting HP-PRRSV cDNA into p3xFlag-CMV-14 (Sigma, St. Louis, MO, USA). The porcine *B2M* promoter was cloned using total DNA extracted from PK-15 cells. DNA fragments containing the porcine *B2M* promoter ranging from nucleotides −1817 to +59 were amplified by PCR using appropriate primers (Table 1), designed according to the upstream sequence of the porcine *B2M* gene (NCBI gene ID [397033](#)). The fragments were inserted into a pGL3 luciferase reporter vector (Promega, Madison, WI, USA) to generate a series of luciferase reporter plasmids, including −1817 B2M, −318 B2M, −218 B2M, −181 B2M, and −163 B2M. A deletion mutant of the luciferase reporter plasmid −1817 Δ PAM B2M, from which the enhancer PAM element of the porcine *B2M* promoter was deleted, was generated by a modified PCR-based site-directed mutagenesis (32). All the above-named plasmids were verified by DNA sequence analysis. For transfection, cells precultured with appropriate media were transfected using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's protocol.

ChIP assay. A chromatin immunoprecipitation (ChIP) assay was performed using an EZ-ChIP chromatin immunoprecipitation kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, PK-15 or Vero cells precultured on 150-mm dishes were transfected with the plasmids indicated in Fig. 5 and incubated at 37°C for 24 h. Cells were fixed by the addition of 275 μ l of 37% formaldehyde into 10 ml of medium, followed by incubation at room temperature for 10 min. Glycine was added at a concentration of 125 mM and incubated at room temperature for 5 min to quench unreacted formaldehyde. The fixed cells were washed twice with cold PBS containing the protease inhibitor cocktail supplied with the kit. The cell pellet was collected by centrifugation and resuspended in 1 ml of lysis buffer (50 mM Tris [pH 8.0], 1% sodium dodecyl sulfate, 10 mM EDTA, and 5 μ g/ml sonicated salmon sperm DNA). The cell lysates were sonicated on ice to shear cross-linked DNA to fragments of about 200 to 1,000 bp in length and centrifuged at 12,000 $\times g$ for 10 min. The supernatants were diluted with the ChIP dilution buffer containing protease inhibitor cocktail and incubated with protein G agarose beads for 1 h. After a brief centrifugation, the supernatants were incubated with anti-Flag monoclonal antibody (M2; Sigma) or normal mouse IgG overnight at 4°C with rotation. The protein G agarose beads were collected by brief centrifugation and washed sequentially with low-salt immune complex wash buffer, high-salt immune complex wash buffer, LiCl immune complex wash buffer, and the Tris-EDTA (TE) buffer supplied with the kit. The washed protein G agarose beads were incubated with 200 μ l of elution buffer at room temperature for 15 min and centrifuged at 4,000 $\times g$ for 1 min. The supernatants were incubated with 5 μ l of 5 M NaCl at 65°C for 4 h, followed by incubation with 1 μ l RNase A at 37°C for 30 min to free the DNA from the protein-DNA complexes. The supernatants were further mixed with 4 μ l of 0.5 M EDTA, 8 μ l of 1 M Tris-HCl, and 1 μ l of proteinase K and incubated at 45°C for 2 h. The DNA was finally purified using the spin columns supplied with the kit. The bound target DNA fractions were detected by PCR with appropriate primers (Table 1). The PCR products were analyzed by 4% agarose gel electrophoresis.

Luciferase assay. PK-15 cells precultured on 24-well plates were transfected with a combination of the luciferase reporter plasmids containing the porcine *B2M* promoter, control plasmid *Renilla* luciferase pRL-TK (Promega), and a plasmid expressing Flag-tagged viral proteins and incubated at 37°C. The transfectants were collected 24 h posttransfection for analysis of firefly luciferase activity using a

TABLE 1 Sequences of primers used

Name	Primer	Primer sequence (5' to 3')
B2M	Forward	CCCGAAGGTTTCAGGTTTA
	Reverse	CCAGATACATAGCAGTTCAG
SLA-I HC	Forward	AGCCATCTTTCCAGTCTAC
	Reverse	CAGTGACCACAGTTCCAA
PRRSV N	Forward	ATCCAGACTGCCTTCAAT
	Reverse	AACTCCACAGTGTAACATTATC
ChIP	Forward	TCCAACCTTTGCCTTCCTA
	Reverse	CCGTCACCCGTCTCTTAG
GAPDH	Forward	TCTGGCAAAGTGGACATT
	Reverse	GGTGAATCATACTGGAACA
MHC-I HC	Forward	GATGTGGAGGAGGAAGAG
	Reverse	TTTACAAGCCGTGAGAGA
–1817 B2M	Forward	TAGGTAGAGCCCGTAG
	Reverse	AGGCCAGACAGTGAGA
–318 B2M	Forward	CGTTGGTGCAAACCTGC
	Reverse	AGGCCAGACAGTGAGA
–218 B2M	Forward	CAGTCCAACCTTTGCC
	Reverse	AGGCCAGACAGTGAGA
–181 B2M	Forward	AGACTTAGAAAATGAA
	Reverse	AGGCCAGACAGTGAGA
–163 B2M	Forward	TGAAACTGAAAACGGG
	Reverse	AGGCCAGACAGTGAGA

dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol and normalized to *Renilla* luciferase activity.

qRT-PCR. Total RNA was isolated from cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions and transcribed into cDNA using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Kyoto, Japan). PCR was performed using SYBR Premix *Ex Taq* (TaKaRa) according to the manufacturer's protocol. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primer sequences are shown in Table 1. Relative mRNA levels of each gene were normalized to GAPDH levels. Fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (33), where *CT* is the cycle threshold. Data are presented as fold changes in gene expression normalized to the GAPDH expression level and relative to levels in mock-infected/treated controls.

Immunofluorescence and Western blotting. Immunofluorescence and Western blotting were performed as described previously (34). The antibodies used were anti-MHC-I monoclonal antibody (1:1,000, W6/32; Santa Cruz), anti- β 2M monoclonal antibody (1:1,000; Abcam, Shanghai, China), anti-GAPDH monoclonal antibody (1:2,000; Proteintech, Chicago, IL, USA), anti-Flag monoclonal antibody (1:1,000, M2; Sigma), rabbit anti-lamin A/C antibody (1:200; Santa Cruz), goat anti-mouse IgG-FITC antibody (1:400; Santa Cruz), and Alexa Fluor 594-conjugated anti-rabbit IgG antibody (1:400; Thermo Fisher Scientific).

Statistical analysis. All experiments were carried out in triplicate. The measured values are expressed as the means with standard deviations (SD). Significance was analyzed using Student's *t* tests. A *P* value of <0.05 was considered significant.

ACKNOWLEDGMENTS

This study was supported by the National Basic Research Program (973 Program grant 2014CB542702), the National Natural Science Foundation of China (grant 31502046), the Chinese Academy of Agricultural Sciences (grant 2015ZL065), and the LinkTADs Project (grant FP7-613804).

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