Porcine Epidemic Diarrhea Virus Infection Induces Caspase-8-Mediated G3BP1 Cleavage and Subverts Stress Granules To Promote Viral Replication

Liumei Sun,^a Huan Chen,^a Xin Ming,^a Zongyi Bo,^a Hyun-Jin Shin,^b Yong-Sam Jung,^a Yingjuan Qian^{a,c}

^aMOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu, China

^bCollege of Veterinary Medicine, Chungnam National University, Daejeon, South Korea

^cJiangsu Agri-Animal Husbandry Vocational College, Veterinary Biopharmaceutical, Jiangsu Key Laboratory for High-Tech Research and Development of Veterinary Biopharmaceuticals, Taizhou, Jiangsu, China

ABSTRACT Porcine epidemic diarrhea virus (PEDV) is an α -coronavirus causing severe diarrhea and high mortality rates in suckling piglets and posing significant economic impact. PEDV replication is completed and results in a large amount of RNA in the cytoplasm. Stress granules (SGs) are dynamic cytosolic RNA granules formed under various stress conditions, including viral infections. Several previous studies suggested that SGs were involved in the antiviral activity of host cells to limit viral propagation. However, the underlying mechanisms are poorly understood. This study aimed to delineate the molecular mechanisms regulating the SG response to PEDV infection. SG formation is induced early during PEDV infection, but as infection proceeds, this ability is lost and SGs disappear at late stages of infection (>18 h postinfection). PEDV infection resulted in the cleavage of Ras-GTPase-activating protein-binding protein 1 (G3BP1) mediated by caspase-8. Using mutational analysis, the PEDV-induced cleavage site within G3BP1 was identified, which differed from the 3C protease cleavage site previously identified. Furthermore, G3BP1 cleavage by caspase-8 at D168 and D169 was confirmed *in vitro* as well as *in vivo*. The overexpression of cleavage-resistant G3BP1 conferred persistent SG formation and suppression of viral replication. Additionally, the knockdown of endogenous G3BP1 abolished SG formation and potentiated viral replication. Taken together, these data provide new insights into novel strategies in which PEDV limits the host stress response and antiviral responses and indicate that caspase-8-mediated G3BP1 cleavage is important in the failure of host defense against PEDV infection.

IMPORTANCE Coronaviruses (CoVs) are drawing extensive attention again since the outbreaks of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019. CoVs are prone to variation and own the transmission capability by crossing the species barrier resulting in reemergence. How CoVs manipulate the antiviral responses of their hosts needs to be explored. Overall, the study provides new insight into how porcine epidemic diarrhea virus (PEDV) impaired SG assembly by targeting G3BP1 via the host proteinase caspase-8. These findings enhanced the understanding of PEDV infection and might help identify new antiviral targets that could inhibit viral replication and limit the pathogenesis of PEDV.

KEYWORDS caspase-8, G3BP1, PEDV, stress granules, viral replication

Coronaviruses (CoVs), belonging to the *Coronaviridae* family in the *Nidovirales* order, are enveloped viruses with a large single-stranded positive-sense RNA. The *Coronaviridae* family includes two subfamilies: *Coronavirinae* and *Torovirinae*. The *Coronavirinae* subfamily is further divided into four genera, α -coronavirus, β -coronavirus,

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Address correspondence to Yong-Sam Jung, ysjung@njau.edu.cn, or Yingjuan Qian, yqian@njau.edu.cn.

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γ -coronavirus, and δ -coronavirus, based on genotypic and serological characterizations (1). The notorious severe acute respiratory syndrome coronavirus (SARS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are β -coronaviruses. Porcine epidemic diarrhea virus (PEDV) is an α -coronavirus. PEDV is the pathogen causing porcine epidemic diarrhea (PED), which is an acute, highly contagious, and severe enteric disease leading to high mortality in suckling piglets and occasional endemics (2, 3). Furthermore, PEDV can recognize protein receptor aminopeptidase N (APN) in humans and infect human cells (4). These pieces of evidence suggest that PEDV poses potential threats to public health concerns. How PEDV and other CoVs manipulate the antiviral responses of their hosts urgently needs to be revealed.

Stress granules (SGs) are dynamic cytosolic RNA granules composed of translationally stalled mRNAs, 40S ribosomes, and various RNA-binding proteins formed in the cytoplasm in response to environmental stresses, including virus infection (5, 6). The functional role of these SGs still remains controversial, but accumulating evidence points to a role of these SGs as a platform for antiviral signal transduction of hosts (5, 7–11). SG contains a stable core structure and a more dynamic shell (12, 13). In general, SGs comprise numerous RNA-binding proteins with an RNA recognition motif (RRM). Among these proteins, Ras-GTPase-activating protein-binding protein 1 (G3BP1) is proposed to be key for the nucleation of SG assembly (14, 15).

Human G3BP1 was first identified by its coimmunoprecipitation with Ras-GTPase-activating protein (RasGAP) (16–18). Although G3BP1 was the first protein shown to bind to the RasGAP SH3 domain, other RasGAP binding proteins have also been reported. Two other reports have shown that G3BP1 can bind to RasGAP during cell proliferation and that the nuclear transfer factor 2 (NTF2)-like domain of G3BP1 is responsible for its interaction (17, 18). However, whether G3BP1 is a genuine RasGAP binding partner is still controversial (19, 20). The C-terminal region of G3BP1 contains two canonical RRM indicating that G3BP1 has an RNA-binding activity. G3BP1 has been characterized as having the activities of endonuclease and helicase (17, 21). It is implicated in Ras signaling, ubiquitin proteasome pathway, RNA processing, and SG assembly (14, 17, 20, 22). These observations suggested a functional role of G3BP1 in the interplay between intra-/extracellular stimuli and mRNA stability. Particularly, G3BP1 is the SG marker protein found in both the SG core and the shell (23). G3BP1 is a tunable switch that triggers phase separation to assemble SGs (24). Previous studies have shown that the overexpression of G3BP1 induces SG formation (14), and the deletion of the RRM domain from G3BP1 abrogates G3BP1-induced SG formation (25).

G3BP1 is composed of five domains: a nuclear transport factor 2-like (NTF2-like) domain, an acidic domain, proline (PxxP) motifs, RRM, and an arginine-glycine (RGG) box. The major functions of the NTF2-like domain are regulation of G3BP1 nuclear transportation (18, 21), dimerization, and SG formation (14). In addition, it is responsible for binding with many proteins, such as RasGAP (18), USP10 (26), caprin-1 (27), OGFOD-1 (28), and proteins containing FGDF motifs (29). The functions of the acidic domain are to increase axonal mRNA translation and disassemble SGs (30). The PxxP domain is essential for recruiting protein kinase R (PKR) to SGs and regulating the antiviral activity of G3BP1 (31, 32). The RRM domain is responsible for G3BP1 endoribonuclease activity (17), and the RGG domain is responsible for G3BP1 DNA and RNA helicase activity (21). The RRM and RGG domains are two RNA-binding regions involved in RNA recognition and the turnover of host mRNA.

SG assembly/disassembly during virus infection is observed in many viruses. Different viruses disrupt SG-related proteins by various of approaches, suggesting that SGs play antiviral roles against these viruses (25, 33–39). In many cases, the disruption of G3BP1 is an important determinant for SG disassembly and viral replication (40). Many studies have shown that G3BP1 is cleaved during viral infection, resulting in SG disassembly (25, 41–48). For instance, poliovirus (PV) infection resulted in G3BP1 cleavage by 3C protease and loss of SG assembly ability. The overexpression of a cleavage-resistant G3BP1 restored SG assembly during PV infection and significantly inhibited

viral replication (25). To date, virus-induced SGs are considered an indication of an antiviral innate response that restricts the translation of the viral genes (5, 10). Many viruses have evolved several strategies to evade the antiviral effect of SGs by degrading and/or sequestering the key components of the antiviral innate response such as G3BP1 to prevent SG formation (25, 40, 44, 49). SG formation can be modulated by coronavirus infection. For example, infectious bronchitis virus (IBV) and transmissible gastroenteritis virus (TGEV) can induce SGs in the late stage of infection (50, 51). Middle East respiratory syndrome coronavirus (MERS-CoV) can inhibit SGs via accessory protein 4a and lead to efficient viral replication (52). PEDV can also induce transient SG formation. However, whether PEDV has also evolved strategic mechanisms to modulate SG formation is unknown thus far (53).

This study investigated the relationship between PEDV and SGs. It demonstrated that PEDV infection caused an initial induction of SGs and then dispersed SG during the late stage of viral replication. It also showed that G3BP1 could be cleaved at D168 and D169 by caspase-8 *in vitro* and *in vivo*. SG assembly was inhibited by caspase-8-mediated G3BP1 cleavage and could be rescued by the overexpression of cleavage-resistant G3BP1. In summary, these results established that the inhibition of caspase-8 activation protected the cells from PEDV infection, suggesting that the balancing between G3BP1 cleavage and SG assembly by caspase-8 modulated the cellular stress threshold.

RESULTS

SGs were induced transiently upon PEDV infection. SGs were observed in cells infected with PEDV (53). However, the dynamics of SGs during PEDV infection and the exact mechanism of PEDV-induced SG formation were still unknown. This study examined SGs on PEDV infection in Vero E6 cells to investigate the extent and kinetics of SGs during PEDV infection. Additional stress agents were not applied to the cell to induce SG formation so we could detect the PEDV-induced SGs during viral infection. G3BP1 is a marker of SG and is found in both the SG core and the shell (23). Therefore, the immunofluorescence analysis of G3BP1 was performed to detect SG formation. Vero E6 cells were stained with antisera specific for PEDV-N protein to detect viral infection and with anti-G3BP1 antibody to detect SGs. Control cells were treated with polyinosinic-polycytidylic acid [poly(I:C)] as a positive control. At the indicated time points, the cells were examined by immunofluorescence microscopy to calculate the percentage of cells displaying G3BP1-positive SGs in PEDV-infected cells. The percentage of cells displaying SGs in PEDV-infected cells gradually increased during the early stage of infection, reaching the maximum level at 18 h postinfection (hpi). SGs were observed to significantly diminish after 18 hpi, indicating that SGs were inhibited during the late stages of viral infection (Fig. 1A). The percentage of cells displaying SGs in PEDV-infected cells was found to be between 3.26 and 14.64% in Vero E6 cells (Fig. 1B). The infected cells were monitored to rule out the cell death effect on SG disassembly in the late stage of infection. The decline in SG formation in the late stage of infection was not likely due to the massive cell death (data not shown). These data suggested that PEDV infection induced SG formation in the early stage of infection and subsequently inhibited it.

PEDV replication was inhibited by SGs. Previous studies have shown that SG assembly can be dominantly induced by the transient overexpression of enhanced green fluorescent protein (EGFP)-G3BP1 without any additional stresses (14) and SG assembly can be inhibited by the knockdown of G3BP1 (54). This study showed that PEDV infection induced SG formation. Thus, whether G3BP1 could regulate PEDV transcription and replication was examined. Vero E6 cells were transfected with G3BP1 or G3BP1 siRNA and then infected with PEDV. The levels of PEDV-N mRNA decreased upon G3BP1 overexpression (Fig. 2A). Also, the expression of PEDV-N protein decreased upon G3BP1 overexpression (Fig. 2B). To confirm the results, the effect of G3BP1 knockdown on PEDV replication was analyzed. Vero E6 cells were transfected with G3BP1 small interfering RNA (siRNA), revealing that the levels of PEDV-N mRNA increased in

FIG 1 SGs were induced transiently upon PEDV infection. (A) Vero E6 cells were transfected with poly (I:C) (2 μ g/ml) for 12 h and mock infected or infected with SQ2014 (1 MOI) for 6, 12, 18, 24, and 36 h. The cells were fixed and stained with G3BP1 antibody (A302-033A) and Alexa 488-conjugated goat anti-mouse IgG antibodies and then stained with PEDV-N antibody and Alexa 555-conjugated goat anti-rabbit IgG antibody. The nuclei were stained with DAPI. The images were acquired with a Nikon confocal microscope. (B) Graph shows the percentage of SG formation in PEDV-infected cells (about 200 cells). The means and standard deviations of three independent experimental replicates are shown. The results were analyzed using the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Vero E6 cells upon G3BP1 knockdown (Fig. 2C). Next, the PEDV-N protein level was determined by Western blotting. The result also showed that the levels of PEDV-N protein were significantly upregulated by G3BP1 knockdown (Fig. 2D). Finally, the viral titers were also determined after the overexpression or depletion of G3BP1. The titers decreased by G3BP1 overexpression (Fig. 2E) and increased when the knockdown of G3BP1 occurred (Fig. 2F). These data suggested that G3BP1 inhibited PEDV transcription and replication.

PEDV disrupted G3BP1-induced SG assembly by cleaving G3BP1. G3BP1 is one of the key SG-nucleating components, considered the SG marker protein. Previous studies showed that SG disassembly occurred by G3BP1 cleavage by viral protease

FIG 2 The replication of PEDV was inhibited by SG formation. A total of 5×10^5 cells were seeded in each well of six-well plates after 18 h and transfected with pEGFP-G3BP1 or empty vector for 24 h, followed by SQ2014 infection (0.01 MOI) for 24 h. N, G3BP1, and actin were detected by using RT-PCR (A) and immunoblotting (B), and virus titers were measured by plaque formation assay (E). A total of 3×10^5 cells were seeded in each well of six-well plates after 18 h and transfected with nontargeting scrambled siRNA or targeting G3BP1 siRNA for 48 h, followed by infection with SQ2014 (0.01 MOI) for 24 h. N, G3BP1, and actin were detected by using RT-PCR (C) and immunoblotting (D), and virus titers were measured by plaque formation assay (F). Con, control.

(25). Therefore, whether the SG disassembly in the late stage of PEDV infection was due to G3BP1 cleavage was investigated. Vero E6 cells were infected with PEDV (SQ2014) for the indicated time points. The whole-cell lysates were subjected to Western blotting using a G3BP1 antibody that could recognize an epitope in the extreme C terminus (amino acids [aa] 416 to 466) of G3BP1. As shown in Fig. 3A, PEDV infection induced G3BP1 cleavage at 24 hpi and a potential cleavage product with 40 kDa apparent mobility. This cleavage product was not detected in Western blotting performed with another antibody that could recognize the N terminus of G3BP1 (data not shown). It was consistently observed that the full-length G3BP1 protein migrated at apparently 68 kDa and the short-form G3BP1 migrated at apparently 40 kDa. Since the short-form G3BP1 was recognized by the G3BP1 antibody that recognized the extreme C terminus of G3BP1, it was named the C-terminal cleavage product of G3BP1 (named G3BP1_{cpc}). Vero E6 cells were infected with two virulent strains (HLJBY and SQ2014) and the vaccine strain CV777 to rule out a potential PEDV strain-specific effect on G3BP1 cleavage. The whole-cell lysates were collected at 24 and 36 hpi. Similarly, the level of G3BP1_{cpc} was detected at 24 hpi and a large amount of G3BP1_{cpc} was detected at 36 hpi in SQ2014, HLJBY, and CV777 infected cells (Fig. 3B).

G3BP1 bearing an N-terminal 2×Flag tag and C-terminal hemagglutinin (HA) tags was overexpressed in cells, and then the cells were infected with PEDV to examine whether the cleavage fragment was derived from N or C termini (Fig. 3C). The examination of Flag Western blots revealed full-length G3BP1 (68 kDa) and 27-kDa cleavage band. Consistent with these data, the study also found that full-length G3BP1 (68 kDa) and 40-kDa cleavage band were detected by HA Western blotting. These findings indicated that the 40-kDa cleavage product contained the C terminus (Fig. 3D).

Two G3BP genes exist. G3BP1 is implicated in SG formation, and its antiviral effects have been extensively studied. However, G3BP2 still remains less well characterized. G3BP1 and G3BP2 share a similar domain structure (55). Like G3BP1, G3BP2 is also important for SG formation, and G3BP1 and G3BP2 appear to have a compensatory function (26). Although 3C protease cleavage sites (E284, Q325, and E405) are highly conserved in G3BP2, the caspase-8 cleavage site (D168 and D169) is not conserved in G3BP2. A potential caspase-8 cleavage site was not detected elsewhere by investigating G3BP2 amino acid sequences (Fig. 3E). Also, G3BP2 could not be cleaved upon PEDV infection (Fig. 3F).

FIG 3 G3BP1 was cleaved in the late stage of PEDV infection. (A) Vero E6 cells were mock infected or infected with SQ2014 (1 MOI) for 6, 12, 18, 24, and 36 h. Whole-cell extracts were prepared and the protein levels of G3BP1, PEDV-N, and actin were analyzed by immunoblotting. The C-terminal cleavage products of G3BP1 (designated G3BP1_{cpc}) are presented with arrows. (B) Vero E6 cells were mock infected or infected with three different strains of PEDV (CV777, HLJBY, and SQ2014) at 1 MOI for 24 or 36 h. Whole-cell extracts were prepared, and the protein level of G3BP1 was analyzed using immunoblotting with the G3BP1 antibody (A302-034A). (C) Schematic presentation for recombinant G3BP1 and G3BP1 antibody epitope (A302-034A). (D) Vero E6 cells were transfected with pcDNA3-2xFlag-G3BP1-HA (1 μ g) for 24 h, and then the cells were mock infected or infected with SQ2014 (1 MOI) for 24 h and 36 h. Whole-cell lysates were prepared and analyzed by immunoblotting. G3BP1 and the N-terminal cleavage products of G3BP1 (designated G3BP1_{cpc}) were detected by Flag

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Caspase-8 proteolytically cleaved G3BP1. Previous studies showed that G3BP1s were cleaved by viral protease protein 3C protease (3C^{pro}) and L protease (L^{pro}) (25, 47). PEDV produces two proteases (Nsp3 papain-like protease and Nsp5 3C-like protease) (56). Nsp5 was overexpressed in Vero E6 and 293T cells to determine whether Nsp5 3C-like protease could cleave G3BP1. G3BP1 cleavage products could not be detected upon Nsp5 overexpression (data not shown). Considering that G3BP1 cleavage occurred in the late stage of PEDV infection and foot-and-mouth disease virus (FMDV) and PV 3C^{pro}-mediated G3BP1 cleavage occurred in the early stage of infection, it was speculated that G3BP1 cleavage in the late stage of PEDV infection was not due to the viral protease. In addition, previous studies showed that apoptosis was induced in the late stage of PEDV infection, and caspase-8 was activated during PEDV infection (57, 58). The activation status of caspase-8 was monitored by Western blotting to determine whether caspase-8 could be activated by PEDV infection. Caspase-8 and cleaved caspase-8 (p43 and p18) were detected at 18 hpi (Fig. 4A). The level of PARP1 cleavage was gradually increased as the level of activated caspase-8 progressively increased (Fig. 4A).

Next, whether activated caspase-8 is required for G3BP1 cleavage was examined. To test this, Vero E6 cells were treated with caspase-8 inhibitor z-IETD-fmk. Vero E6 cells were pretreated with z-IETD-fmk for 1 h before viral infection, followed by PEDV infection for another 36 h. The immunoblot analysis showed that G3BP1 cleavage gradually decreased in z-IETD-fmk-treated Vero E6 cells in a dose-dependent manner (Fig. 4B). The C-terminal Flag-tagged G3BP1 (G3BP1-Flag) was transiently transfected into Vero E6 cells along with an increased dose of caspase-8-HA to confirm G3BP1 cleavage. Caspase-8 overexpression resulted in G3BP1-Flag protein cleavage, and a 40-kDa G3BP1-Flag cleavage product appeared in a dose-dependent manner (Fig. 4C). The 40-kDa migration of the cleavage fragment containing a C-terminal Flag suggested that G3BP1 cleavage occurred closer to the N terminus than to the C terminus of the G3BP1.

In vitro enzyme digestion experiments were performed to investigate whether caspase-8 could cleave G3BP1 directly. Purified G3BP1 and caspase-8 were prepared with a nickel column purification system. Purified G3BP1 were incubated *in vitro* along with different concentrations of purified caspase-8. The reaction mixture was analyzed by SDS-PAGE and Western blotting. The result showed that caspase-8 caused G3BP1 cleavage (Fig. 4D). Only two obvious cleavage products were observed, migrating with apparent mobility patterns of 40 kDa and 27 kDa, suggesting that a single cleavage site was present within the G3BP1 sequence (Fig. 4D). The G3BP1 antibody could detect a 40-kDa G3BP1 cleavage product but not a 27-kDa G3BP1 cleavage product (Fig. 4E). Western blotting revealed full-length G3BP1 and 40-kDa bands, indicating that the 40-kDa cleavage product comprised the C terminus of G3BP1. These data indicated that G3BP1 could be cleaved by caspase-8 during PEDV infection.

Caspase-8 cleaved G3BP1 at Asp168 and Asp169. The study next searched for the potential caspase-8 cleavage sites in the sequence of G3BP1. Based on the data, it focused on the N-terminal sequence of G3BP1 for potential caspase-8 cleavage sites at which scissions could lead to produce ~40-kDa fragments. A series of G3BP1 mutants were constructed in which aspartic acid (Asp, D) was substituted with glutamic acid

FIG 3 Legend (Continued)

antibody. G3BP1 and G3BP1_{cpc} were detected using HA and G3BP1 antibody (A302-034A). The arrows denote the positions of all G3BP1 bands. (E) Schematic presentation of amino acid sequence alignment of G3BP1, G3BP2a, and G3BP2b (performed using Clustal Omega [<https://www.ebi.ac.uk/Tools/msa/clustalo/>]). Sequences and NCBI accession numbers used in this alignment were as follows: G3BP1 (NP_005745.1), G3BP2a (NP_036429.2), and G3BP2b (NP_987100.1). (F) Vero E6 cells were mock infected or infected with SQ2014 (1 MOI) for 12, 24, and 36 h. Whole-cell extracts were prepared and analyzed by immunoblotting. G3BP2a and G3BP2b were detected using the G3BP2 antibody. Asterisks indicate positions which have a single, fully conserved residue. A colon indicates conservation between groups of strongly similar properties. A period indicates conservation between groups of weakly similar properties.

FIG 4 G3BP1 was cleaved by caspase-8. (A) Vero E6 cells were mock infected or infected with SQ2014 (1 MOI) for 6, 12, 18, 24, and 36 h. Whole-cell extracts were prepared and analyzed by immunoblotting with the indicated protein antibodies. (B) Vero E6 cells were treated with the caspase-8 inhibitor Z-IETD-fmk (10, 20, and 50 μ M) or an equivalent volume of dimethyl sulfoxide (DMSO) for 1 h, and then the cells were mock infected or infected with SQ2014 (1 MOI) for 36 h. Whole-cell extracts were prepared and analyzed by immunoblotting with the indicated protein antibodies. (C) Vero E6 cells were transfected with pcDNA4-G3BP1-2 $\times$ Flag (1 μ g), along with increasing quantities of pcDNA4-caspase-8-HA (0, 25, 75, and 125 ng) for 24 h. Whole-cell lysates were prepared and analyzed by immunoblotting. G3BP1 was detected by the Flag antibody, and caspase-8 was detected by the HA antibody. Purified recombinant His-tagged G3BP1 was incubated with or without the purified recombinant caspase-8 (0, 1, and 10 μ g) at 37°C for 4 h, and the reaction products were analyzed by SDS-PAGE (D) or immunoblotting with the G3BP1 antibody (E). The positions of N-terminal cleavage products of G3BP1 (designated G3BP1_{cpn}) and C-terminal cleavage products of G3BP1 (designated G3BP1_{cpc}) are indicated by arrows.

(Glu, E) (Fig. 5A). A total of seven G3BP1 mutants were generated which carried single or double substitutions with Glu at D129, D135, D168, D169, D175, and D182 along with a His or green fluorescent protein (GFP) tag (Fig. 5A). Purified His-G3BP1 wild-type (G3BP1 WT) and mutant proteins were incubated with caspase-8 protein *in vitro*, and the reaction mixture was analyzed using Coomassie blue staining and Western blotting. Four G3BP1 mutants, which carried Glu substitution (D129E, D135E, D175E, and D182E), were still found to be cleaved by caspase-8 (Fig. 5B). In contrast, the D168E (M3) and D168E (M4) blocked the caspase-8-mediated cleavage, yielding only full-length G3BP1 protein in the presence of caspase-8 (Fig. 5B). A similar result was observed in Western blotting (Fig. 5C). Three G3BP1 mutants, which carried single and

FIG 5 G3BP1 was cleaved by caspase-8 at Asp168 and Asp169. (A) Schematic presentation of G3BP1 mutations. Purified wild-type or mutants of His-tagged G3BP1 were incubated with or without the purified recombinant caspase-8 (10 μ g) at 37°C for 4 h, and the products were analyzed by SDS-PAGE (B) or immunoblotting with the G3BP1 antibody (C). (D) Vero E6 cells were transfected with wild-type or mutants of EGFP-tagged G3BP1 for 24 h and then mock infected or infected with SQ2014 (1 MOI) for 24 h. G3BP1, PEDV-N, and actin were detected by immunoblotting. (E) Schematic presentation of G3BP1 cleavage sites. Porcine G3BP1 (E284) corresponds to the human G3BP1 (E285).

double substitutions with Glu at D168, D169, and D168/169 along with a GFP tag, were generated to confirm that G3BP1 was cleaved at D168 and D169 upon PEDV infection. These mutants were then expressed in Vero E6 cells and infected with SQ2014 for 24 h. We found that WT, M3, and M4 were still cleaved (Fig. 5D). In contrast, G3BP1 cleavage was significantly decreased for the G3BP1 mutant with a double substitution of Asp with Glu (D168/169E, referred to here as M7) (Fig. 5D). These results suggested that caspase-8 proteolytically cleaved G3BP1 at one of the two Asp residues (D168 and D169). Previous studies showed that G3BP1 was cleaved by viral protease at different sites. As shown in Fig. 5E, PV, encephalomyocarditis virus (EMCV), coxsackievirus type B3 (CVB3), and human enterovirus D68 (EV-D68) 3C protease cleaved G3BP1 at 325 (25, 41, 48), feline calicivirus (FCV) 3C-like proteinase cleaved G3BP1 at E405 (44), and FMDV 3C protease cleaved G3BP1 at E284 (46). The PEDV-induced cleavage site was identified within G3BP1, which differed from the previously identified 3C protease cleavage site (Fig. 5E). Porcine G3BP1 (E284) corresponded to the human G3BP1 (E285) (Fig. 5E).

Cleavage-resistant G3BP1 restored SG formation during PEDV infection. A previous study showed that the overexpression of G3BP1 induced SG formation (14), and the deletion of the G3BP1 RRM domain from the known protein-interaction motif destroyed G3BP1-induced general SG formation (25, 44, 47). Vero E6 cells were transfected with GFP-tagged G3BP1 (WT), M3, M4, or M7 24 h before viral infection was initiated to examine whether the expression of cleavage-resistant G3BP1 altered SG formation during PEDV infection. Followed by PEDV infection for 24 h, Vero E6 cells were stained with antisera specific for the PEDV-N protein, and the SG formation was

FIG 6 SGs were rescued in Vero E6 cells expressing cleavage-resistant G3BP1. (A) Vero E6 cells were transfected with WT (1 μ g), M3 (1.2 μ g), M4 (1.2 μ g), and M7 (1.2 μ g) for 24 h and then mock infected or infected with SQ2014 (1 MOI) for 24 h. Then, the cells were fixed and stained with PEDV-N antibody and Alexa 555-conjugated goat anti-rabbit IgG antibody (red). The nuclei were stained with DAPI (blue). Images were acquired with a Nikon immunofluorescence microscope. (B) Graph shows the percentage of cells containing SGs (counting 200 cells). The means and standard deviations of three independent experimental replicates are shown. (C) Vero E6 cells were treated with Z-VAD-fmk (20 μ M), Z-IETD-fmk (20 μ M), or an equivalent volume of dimethyl sulfoxide (DMSO) for 1 h, and then the cells were infected with SQ2014 (1 MOI) for 0, 24, and 36 h. The cells were fixed and stained with G3BP1 antibody (A302-033A) and PEDV-N antibody. The nuclei were stained with DAPI. The images were acquired with a Nikon confocal microscope. (D) Graph shows the percentage of SG formation in PEDV-infected cells (about 200 cells). The means and standard deviations of three independent experimental replicates are shown.

monitored by the fluorescence of EGFP-G3BP1. The immunofluorescence microscopic analysis revealed that the cells transfected with G3BP1 WT, M3, M4, and M7 were almost equally able to form SGs (Fig. 6A, uninfected column). However, the cells transfected with G3BP1 WT, M3, and M4 were not able to form SG upon PEDV infection (Fig. 6A, infected column). Importantly, cleavage-resistant G3BP1 (M7)-transfected cells maintained their ability to form SGs (Fig. 6A). The percentage of SGs in PEDV-positive cells was also calculated to confirm the ratio of SGs in cells. Upon PEDV infection, the

FIG 7 Replication of PEDV was inhibited by cleavage-resistant G3BP1. Vero E6 cells were transfected with WT (1 μ g), M3 (1.2 μ g), M4 (1.2 μ g), and M7 (1.2 μ g) for 24 h and then infected with SQ2014 (0.01 MOI) for 24 h. PEDV-N, G3BP1, and actin were detected by semiquantitative RT-PCR (A) and immunoblotting (B), and virus titers were detected by plaque formation assay (C). ***, $P < 0.001$. (D) Vero E6 cells were treated with Z-VAD-fmk (20 μ M), Z-IETD-fmk (20 μ M), or an equivalent volume of dimethyl sulfoxide (DMSO) for 1 h, and then the cells were infected with SQ2014 (1 MOI) for 0, 24, and 36 h. Whole-cell extracts were prepared and analyzed by immunoblotting with the indicated antibodies.

cells transfected with G3BP1 WT, M3, and M4 quickly lost the ability to form SGs, whereas cleavage-resistant G3BP1 (M7)-transfected cells formed a significant number of SGs (Fig. 6B). To confirm that SGs were disrupted during PEDV infection due to G3BP1 cleavage by caspase-8, the cells were treated with pan-caspase (Z-VAD-fmk) and caspase-8 (Z-IETD-fmk) inhibitors in the late stage of PEDV infection and the cells were examined by immunofluorescence microscopy to calculate the percentage of cells displaying G3BP1-positive SGs in PEDV-infected cells. The results showed that SGs were significantly enhanced in cells treated with pan-caspase and caspase-8 inhibitors (Fig. 6C). The percentage of cells displaying SGs in PEDV-infected cells was increased in cells treated with pan-caspase and caspase-8 inhibitors (Fig. 6D). These results suggested that intact G3BP1 was required for SG formation and that the cleavage by caspase-8 was sufficient to block the ability of SG formation *in vivo*.

Cleavage-resistant G3BP1 negatively regulated PEDV replication. The overexpression of G3BP1 WT induced SG formation and inhibited PEDV replication. Recent studies showed that SG formation was an important aspect of the antiviral response (5, 7–11, 31, 32, 59, 60). Indeed, cleavage-resistant G3BP1 (M7) maintained SGs during PEDV infection. These data led us to investigate whether G3BP1 cleavage was beneficial for viral replication in cells. To test this, Vero E6 cells were transfected with G3BP1 WT, M3, M4, or M7 and then infected with SQ2014 PEDV. The levels of viral N mRNA dramatically decreased by cleavage-resistant G3BP1 (M7) overexpression (Fig. 7A). Also, the expression of viral N protein decreased on cleavage-resistant G3BP1 (M7) overexpression (Fig. 7B). The viral titers were determined upon overexpression of G3BP1 mutants in Vero E6 cells to confirm the effect of cleavage-resistant G3BP1 (M7) on viral replication. The viral titers significantly decreased by cleavage-resistant G3BP1 (M7) overexpression (Fig. 7C). These data suggested that G3BP1 inhibited PEDV transcription and replication via SG assembly. To confirm that G3BP1 cleavage-induced SG disassembly can promote the PEDV replication, pan-caspase and caspase-8 inhibitors were used to treat the SQ2014-infected cells at 24 hpi and 36 hpi, and the PEDV-N level was checked using Western blotting. The result showed that the PEDV-N level

FIG 8 Model for the interplay between caspase-8 and G3BP1-containing SGs during PEDV infection. The host cells sensed the infection of PEDV and induced G3BP1 dimerization/oligomerization and SG formation. PEDV infection activated caspase-8 and subsequently led to G3BP1 cleavage to counteract the inhibitory effect of SGs. PEDV infection induced apoptosis through caspase-8 activation.

decreased obviously in cells treated with pan-caspase and caspase-8 inhibitors (Fig. 7D). The data suggested that PEDV replication was inhibited when SG disassembly was blocked through the inhibition of caspase-8-induced G3BP1 cleavage with caspase inhibitors. These results suggested that caspase-8-mediated G3BP1 cleavage was required for PEDV replication and that the SG formation could be sufficient to inhibit PEDV replication.

As illustrated in Fig. 8, SGs were induced transiently in the early stage of PEDV infection. Also, caspase-8 could be activated and subsequently led to G3BP1 cleavage in the later stage of PEDV infection. Further, cleaved G3BP1 could have lost the capability of SG formation and could have also lost the antiviral activity. Collectively, it was speculated that G3BP1 cleavage was essential for PEDV replication.

DISCUSSION

PED is a contagious viral disease in pigs caused by PEDV. PEDV infection results in significant mortality in piglets. Like that of many other RNA viruses, the viral genome of PEDV is a functional mRNA that must be efficiently translated in the early stage of the infectious cycle. This study characterized the posttranslational modification of G3BP1 in PEDV-infected cells to elucidate the stress responses and critical host factors regulated by the virus, aiming to understand PEDV pathogenesis. PEDV infection induced SG formation transiently and then later caused SG disassembly. At 24 hpi, the capability of cells to form SGs was dramatically suppressed and was almost gone in the late stage of infection. The degree of inhibition of SG formation correlated closely with the expression of activated caspase-8 during viral infection, which also correlated with G3BP1 cleavage (D168 and D169). Importantly, SG formation could be rescued by the expression of a cleavage-resistant G3BP1 (M7), suggesting that the full-length G3BP1 was required for SG formation in cells. The data uncovered the novel role of caspase-8 in controlling SGs by G3BP1 cleavage and suggested a novel antiviral mechanism of G3BP1 as a negative regulator of PEDV replication.

Several studies highlighted the importance of viral protease-mediated G3BP1 cleavage as an antagonizing mechanism to inhibit SG formation during viral infections. Previous work showed that the infection with PV, EMCV, CVB, EV-D68, FCV, or FMDV resulted in G3BP1 cleavage and SG disassembly (25, 41, 42, 44, 45, 48). All four viruses (such as PV, EMCV, CVB3, and EV-D68) targeted G3BP1 at the same site (Q325), whereas the 3C-like protease of FCV and FMDV specifically cleaved G3BP1 at E405 and E284,

respectively. The results suggested that caspase-8 cleaved G3BP1 at a position (D168 and D169) that differed from that of the previously identified 3C protease cleavage at E284, Q325, or QE405. Preventing caspase-8-mediated G3BP1 cleavage rescued SG formation and subsequently inhibited PEDV replication through the overexpression of wild-type or cleavage-resistant G3BP1 (Fig. 7). This finding confirmed that G3BP1 cleavage during PEDV infection might act as a major contributor to the inhibition of SG assembly and the promotion of viral replication. Although many viral 3C proteases cleaved off the RRM/RGG motifs in the C terminus of G3BP1, caspase-8 cleaved G3BP1 in the acidic region of G3BP1, targeting the N-terminal domain. Thus, G3BP1-containing SGs could be differentially modulated by various proteases to cleave G3BP1.

SGs are dynamic cytosolic RNA granules. The PEDV replication cycle is completed in the cytoplasm, and a large amount of genomic RNA and minus-strand RNA are produced during PEDV replication (61). In addition, a previous study found that SG marker protein G3BP1 played an antiviral role in PEDV replication, and PEDV titers were decreased by G3BP1 overexpression and increased by G3BP1 knockdown (53). However, the underlying mechanisms were not investigated. Hence, the formation and function of SG in PEDV replication were investigated. This study demonstrated that caspase-8 could be activated by PEDV infection (Fig. 4A). In addition, a caspase-8 inhibitor (Z-IETD-fmk) was used to investigate whether caspase-8 activation was related to G3BP1 cleavage during PEDV infection. The kinetics and extent of SG disassembly correlated closely with both active caspase-8 and G3BP1 cleavage. The cellular stress response is an important mechanism for maintaining cell homeostasis (62). Stress granules and apoptosis are two different cellular stress responses. The apoptotic activity of PEDV viral proteins (such as Nsp1 and S1) and SARS-coronavirus M, N, and 3C-like protease has been revealed (57, 63–65). However, the relationship between SGs and apoptosis has not been investigated yet. The viral targeting of G3BP1 could be more complete in a later stage of PEDV infection. PEDV-induced apoptosis and G3BP1 cleavage might be operated interdependently to disable the role of G3BP1 in SG formation and provide a survival benefit, which needs further investigation.

The antiviral function of SGs could be exerted in two ways. First, SG interacting components recruit numerous antiviral proteins, including retinoic acid-inducible gene I (RIG-1) and PKR, and subsequently enhance the induction of the innate immune response (5, 19). Therefore, it is likely that RIG-1 exerts its antiviral function through facilitating viral RNA sensing in SGs. Second, SGs might also sequester viral mRNA into SGs and thereby suppress the viral mRNA translation. Based on proteomic analysis, 50% of SG components are a subset of RNA-binding proteins (23). DEAD-box helicase 3 (DDX3) is a component of SGs and is postulated to modulate protein translation (11, 66). It is conceivable that through this type of regulation, DDX3 suppresses viral mRNA translation by sequestering the translation initiation complex into the SGs. Because SGs sequester numerous proteins involved in RNA biogenesis and function, the SG formation is likely to have broad effects on viral protein expression. How SG assembly affects the regulation of viral mRNA function remains to be established. This supports the hypothesis that the sequestration of certain cellular proteins within SGs promotes viral infection by disrupting antiviral signaling pathways.

In conclusion, this study provided evidence that PEDV actively suppressed SG formation through the proteolytic activity of caspase-8. It identified the SG core protein G3BP1 as a target of caspase-8. The results also identified caspase-8 as a more dynamic regulator of G3BP1 function and suggested a potential mechanism of SG disassembly via a variety of stress responses through apoptotic molecules. Elucidating how G3BP1 cleavage and SG disassembly are regulated might lead to a better understanding of the role of caspase-8 in the stress responses and apoptosis.

MATERIALS AND METHODS

Cell culture and plasmids. African green monkey *Cercopithecus aethiops* kidney epithelial cells (Vero E6 cells) and human embryonic kidney 293 cells (293T cells) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and

TABLE 1 Primer pairs used for G3BP1 mutagenesis

Name	Sequence
G3BP1-D129E-F	CTATGTTTCAATGAAATCTTCAGATACCAAG
G3BP1-D129E-R	CTTGGTATCTGAAGATTCATTGTGAACATAG
G3BP1-D135E-F	CTTCAGATACCAAGAAGAGGTCTTTGGTG
G3BP1-D135E-R	CACCAAAGACCTCTTCTTGGTATCTGAAG
G3BP1-D168E-F	GAGGTGGTACCTGAGGATTCTGGAACCTTC
G3BP1-D168E-R	GAAAGTTCAGAATCCTCAGGTACCACTC
G3BP1-D169E-F	GTGGTACCTGATGAGTCTGGAACCTTCTATG
G3BP1-D169E-R	CATAGAAAGTTCAGACTCATCAGGTACCAC
G3BP1-D175E-F	GAACTTTCTATGAGCAGGCAGTTGTCTAGTAATG
G3BP1-D175E-R	CATTACTGACAACTGCCTGCTCATAGAAAGTTC
G3BP1-D182E-F	GTTGTCAGTAATGAGATGGAAGAACATTTAG
G3BP1-D182E-R	CTAAATGTTCTTCCATCTCATTACTGACAAAC

streptomycin at 37°C in a 5% CO₂ incubator. The human G3BP1 (GenBank accession number [NM_005754.3](#)) gene was amplified from the cDNA of 293T cells and cloned into pcDNA3-N-2×Flag-C-HA (pcDNA3 vector with N-terminal 2×Flag tag and C-terminal HA tag), pcDNA4/TO-C-2×Flag (pcDNA4/TO vector with C-terminal 2×Flag tag), pEGFP-C1, and pET-30a separately. The human caspase-8 (GenBank accession number [NM_033355.3](#)) (217 to 479 aa) was amplified from the cDNA of 293T cells and cloned into pcDNA4/TO-C-HA (pcDNA4/TO vector with C-terminal HA tag) and pET-30a. PEDV-Nsp5 was amplified from the cDNA of the SQ2014 (GenBank accession number [KP728470.1](#)) virus and cloned into the pcDNA3-2×Flag vector. Replacement of aspartic acid (D) with glutamic acid (E) in G3BP1 was generated by site-directed mutagenesis. The primers used in this study are shown in Table 1.

Antibodies and reagents. Rabbit polyclonal anti-G3BP1 (A302-033A), rabbit polyclonal anti-G3BP1 (A302-034A), and rabbit polyclonal anti-G3BP2 (A302-040A) antibodies were purchased from Bethyl Laboratories, Inc. (TX, USA). Mouse polyclonal anti-PEDV-N and rabbit polyclonal anti-PEDV-N were previously generated in the lab. Alexa 488-conjugated goat anti-mouse IgG (A-11001) antibody and Alexa 555-conjugated goat anti-rabbit IgG (A-21428) antibody were purchased from Thermo Fisher Scientific, Inc. (MA, USA). Rabbit polyclonal anti-actin (A2066) and mouse monoclonal anti-Flag (F1804), a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) (AP307P), and HRP-conjugated goat anti-mouse IgG (H+L) (AP308P) antibodies were purchased from Merck, Inc. (Darmstadt, Germany). Mouse monoclonal anti-caspase-8 (9746S), rabbit polyclonal anti-PARP (9542S), and rabbit monoclonal anti-HA (3724S) antibodies were purchased from Cell Signaling Technology, Inc. (MA, USA). A HiScript 1st strand cDNA synthesis kit (R111-01) was purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). Lipofectamine 2000 (11668027) was purchased from Thermo Fisher Scientific, Inc. (MA, USA). Poly(I:C) with a low molecular weight (tlrl-picw) was purchased from InvivoGen Inc. (CA, USA). The inhibitors used in this study, including pan-caspase (A1902) and caspase-8 inhibitors (B3232), were purchased from APEXIO Technology, Inc. (TX, USA). His-affinity agarose beads (HD1001) were purchased from Pointbio, Inc. (Shanghai, China).

Viral infection and titer determination. PEDV (strains CV777, HJBY, and SQ2014) was propagated in Vero E6 cells. The cells were infected with PEDV in DMEM without FBS and incubated at 37°C for 1 h. After incubation, the cells were washed with phosphate-buffered saline (PBS) and transferred to DMEM containing 2% FBS and 17.5 ng trypsin per ml. The virus titer was determined by the plaque formation assay. Briefly, 10-fold serial dilutions (10³ to 10⁸) of viruses were incubated with a confluent monolayer of Vero E6 cells at 37°C for 1 h. The unbound virus was removed by washing with cold PBS. An overlay medium (2% low-melting-point agarose in DMEM containing 2% FBS and 17.5 ng trypsin per ml) was added to each well, and the plates were incubated at 37°C with 5% CO₂ for 2 to 3 days. The cells were stained with 0.5% crystal violet.

RNA extraction and semiquantitative RT-PCR. Total RNA was isolated from about 1.0 × 10⁶ cells with or without PEDV infection by using TRIzol reagent following the manufacturer's instructions. The cDNA was synthesized using a HiScript 1st strand cDNA synthesis kit following the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) primers were designed by using Oligo (version 6.0) software. The sequences of the primers for viral and cellular gene amplification were as follows: PEDV-N-F (5'-TTG ATG CGT CAG GCT ATG CTC-3') and PEDV-N-R (5'-CAT TCT GCT GCG TGG TTT-3'), G3BP1-F (5'-CAA AGA GTG CGA GAA CAA CGA-3') and G3BP1-R (5'-AAT TTC CCA CCA CTG TTA ATG C-3'), and actin-F (5'-CTG AAG TAC CCC ATC GAG CAC GGC A-3') and actin-R (5'-GGA TAG CAC AGC CTG GAT AGC AAC G-3').

Transient transfection. A total of 5 × 10⁵ Vero E6 cells were seeded into each well of six-well plates for 18 h before transfection. Transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen, USA) at a ratio of 2 μl transfection reagent to 1 μg DNA, and other procedures were also performed following the manufacturer's instructions. Under this condition, at least 50% of cells could be transfected and could express the plasmid DNA transiently. To ensure equal expression level of G3BP1 WT and variants, different dosages of plasmids were transfected at the specific dosages shown in figure legends.

Knockdown of G3BP1 expression. siRNA oligonucleotides against G3BP1 and a nontargeting control siRNA were purchased from Biotend, Inc. (Shanghai, China). For siRNA gene knockdown experiments, 3×10^5 Vero E6 cells were seeded into each well of six-well plates for 18 h before transfection and transfected with small interfering RNA (siRNA) oligonucleotide (50 nM) using Lipofectamine 2000 following the manufacturer's instructions. After 48 h of transfection, the cells were analyzed by immunoblotting to determine the knockdown efficiency. The G3BP1 RNA interference (RNAi) target sequences were as follows: number 1, 5'-CAU UAA CAG UGG UGG GAA A-3', and number 2, 5'-GGA GAU UCA UGC AAA CGU U-3'.

Immunofluorescence microscopy. The cells were grown on coverslips and infected with or without PEDV for different time points. The cells on the coverslips were fixed and permeabilized with 4% formaldehyde and 0.1% Triton X-100 at 37°C for 30 min. After washing with glycine-PBS (0.02 M glycine in PBS), the cells were blocked with 3% bovine serum albumin (BSA) in PBS at 37°C for 30 min. They were incubated with primary antibody at 37°C for 1 h, followed by washing thrice with PBS with Tween 20 (PBST) to remove unbound antibodies. Then, they were incubated with secondary antibody at 37°C for 30 min. Unbound antibodies were removed by washing thrice with PBST. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) containing the anti-fade Dabco solution. The images were obtained under a Nikon fluorescence microscope (TS100-F; DSRI2).

Expression and purification of caspase-8 and G3BP1. *Escherichia coli* BL21 cells containing pET-30a-caspase-8 or pET-30a-G3BP1 (wild type and mutants) plasmids were grown at 37°C in LB medium until an A_{600} reached 0.6 to 0.8 and induced by adding 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) at 16°C for 18 h. The cells were harvested, washed with buffer A (25 mM HEPES [pH 7.5], 150 mM NaCl, and 0.5% NP-40), and lysed by ultrasonication. After centrifugation ($29,820 \times g$ for 30 min), the supernatant was incubated with the His-affinity agarose beads on a rocker at 4°C for 2 h and washed with buffer A supplemented with 10 mM imidazole. Proteins were eluted from the His-affinity agarose beads with buffer A containing 300 mM imidazole.

Cleavage assay *in vitro*. Purified recombinant G3BP1 (2 μ g) was incubated with or without purified recombinant caspase-8 in a total reaction volume of 25 μ l at 37°C for 4 h. Reactions were carried out in a caspase reaction buffer (200 mM HEPES [pH 7.5], 20% sucrose, 0.2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 10 mM dithiothreitol [DTT]). The products were analyzed by SDS-PAGE and immunoblotting.

Statistical analysis. The results were representative of experiments repeated three times. All results were analyzed with GraphPad Prism and are presented as means plus or minus standard deviations (SDs). Statistical significance was determined using the two-tailed Student's unpaired-sample *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Data availability. All the data are fully available without restriction.

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We declare that we have no conflict of interest.

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