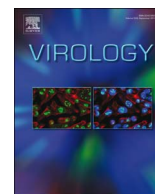




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# Foot-and-mouth disease virus induces lysosomal degradation of host protein kinase PKR by 3C proteinase to facilitate virus replication

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## ABSTRACT

The interferon-induced double-strand RNA activated protein kinase (PKR) plays important roles in host defense against viral infection. Here we demonstrate the significant antiviral role of PKR against foot-and-mouth disease virus (FMDV) and report that FMDV infection inhibits PKR expression and activation in porcine kidney (PK-15) cells. The viral nonstructural protein 3 C proteinase (3C<sup>pro</sup>) is identified to be responsible for this inhibition. However, it is independent of the well-known proteinase activity of 3C<sup>pro</sup> or 3C<sup>pro</sup>-induced shutoff of host protein synthesis. We show that 3C<sup>pro</sup> induces PKR degradation by lysosomal pathway and no interaction is determined between 3C<sup>pro</sup> and PKR. Together, our results indicate that PKR acts an important antiviral factor during FMDV infection, and FMDV has evolved a strategy to overcome PKR-mediated antiviral role by downregulation of PKR protein.

## 1. Introduction

Foot-and-mouth disease virus (FMDV) is an RNA virus belonging to the genus *Aphthovirus* of family *Picornaviridae*. FMDV is the causative agent of foot-and-mouth disease (FMD) that causes highly contagious viral disease of cloven-hoofed animals (Grubman and Baxt, 2004). The outbreak of FMD often results in severe economic consequences due to its impact on trade and the slaughtering of large amounts of animals (Jamal and Belsham, 2013). The genome of FMDV is a positive-strand RNA of ~8.5 kb encoding a large polyprotein. The viral leader proteinase (L<sup>pro</sup>), 2A protein, and 3C proteinase (3C<sup>pro</sup>) subsequently cleaves the polyprotein to yield mature structural and nonstructural proteins (Steinberger et al., 2014). L<sup>pro</sup> and 3C<sup>pro</sup> are multifunctional proteins. In addition to the vital roles in processing of the polyprotein precursor, it is well-known that L<sup>pro</sup> and 3C<sup>pro</sup> are significantly involved in the viral antagonistic activity against host antiviral responses (Lawrence et al., 2012; Liu et al., 2015; Stenfeldt et al., 2016).

L<sup>pro</sup> is widely known to promote virus propagation by disrupting the interferon (IFN) signaling pathway to suppress host innate immune responses (Liu et al., 2015; Steinberger and Skern, 2014). 3C<sup>pro</sup> belongs to the family of chymotrypsin-like cysteine proteases, and plays an important role in FMDV pathogenesis (Curry et al., 2007).

3C<sup>pro</sup> can suppress host antiviral responses by different mechanisms, such as, inducing cleavage of eukaryotic translation initiation factor 4G (eIF4G) to shut off host protein synthesis; cleaving host antiviral proteins and inhibition of IFN- $\alpha/\beta$  production (Du et al., 2014a; Lawrence et al., 2012; Liu et al., 2015). The significant roles of 3C<sup>pro</sup> in viral pathogenesis make 3C<sup>pro</sup> as an attractive target for the design of antiviral agents (Sweeney et al., 2007). The mechanisms about the 3C<sup>pro</sup>-mediated antagonistic effects are further exploited to provide insights for uncovering the viral pathogenesis.

The interferon-induced double-strand RNA activated protein kinase (PKR) plays important roles in host defense against viral infection. PKR is a serine-threonine kinase that constitutively expresses in mammalian cells (Habjan et al., 2009; Nallagatla et al., 2011). As an antiviral factor, PKR can be induced by IFN treatment and is activated by binding of double-stranded RNA (dsRNA) or the PKR activator (PACT) protein. Activation results in PKR phosphorylation and subsequently phosphorylates the  $\alpha$  subunit of the eIF2 translation initiation factor (eIF2 $\alpha$ ), blocks cellular and viral protein synthesis, thereby interfering with viral transcription or translation in infected cells (Nallagatla et al., 2011). To disrupt or antagonize antiviral effects of PKR, various viruses have evolved different strategies to inhibit PKR activation. Influenza A virus non-structural (NS1) protein interacts with PKR

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or directly binds the double-stranded RNA to block PKR activation and counteract PKR-mediated inhibition of viral replication (Bergmann et al., 2000; Li et al., 2006; Lu et al., 1995). Hepatitis C virus (HCV) internal ribosome entry site binds to PKR in competition with PKR ligands and prevents the phosphorylation and activation of PKR (Vyas et al., 2003). Our previous study found that FMDV infection leads to a significant decrease of PKR protein as infection progresses (Li et al., 2016b). It implies that PKR may suppress FMDV infection and the virus decreases PKR protein expression to block PKR activation and antagonize the antiviral response.

In the present study, we examined the FMDV modulation of PKR signaling in porcine cells. Here we report that overexpression of PKR could significantly suppress FMDV replication. FMDV infection led to decreased levels of PKR protein in comparison with that in the mock-infected cells. The viral 3C<sup>pro</sup> was determined to be responsible for this decrease of PKR. 3C<sup>pro</sup>-induced PKR reduction was independent of its proteinase activity and the ability to cleave eIF4G.

## 2. Results

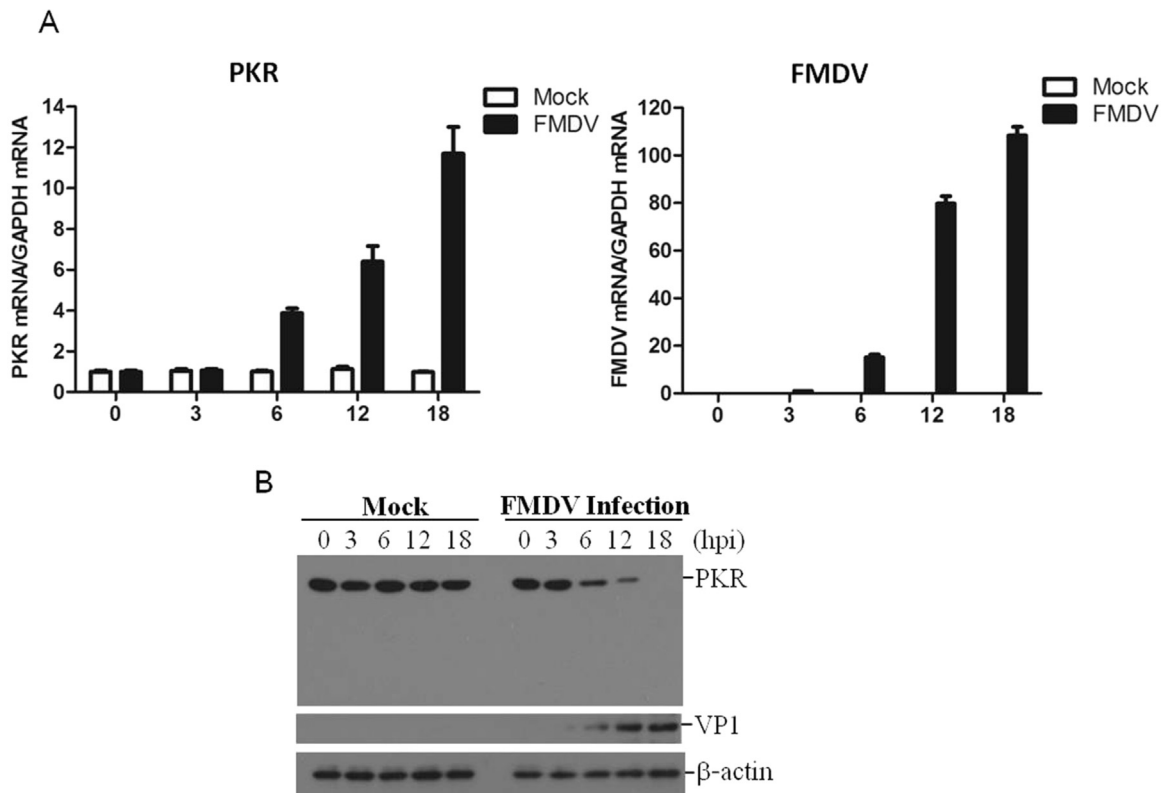
### 2.1. Dynamics of PKR expression in response to FMDV infection

The addition of 2-aminopurine (2-AP), an inhibitor of dsRNA inducible protein kinase (PKR), increases the yield of FMDV in PK-15 cells (Chinsangaram et al., 2001). This indicates the important role of PKR in inhibition of FMDV replication. It was therefore necessary to investigate the state of PKR during FMDV infection. We first investigated the kinetics of PKR to determine the state of PKR in FMDV-infected cells. PK-15 cells were infected by FMDV at an MOI of 0.5, and the dynamics of PKR were examined. It showed that the mRNA level of PKR was significantly upregulated starting at 6 h post-infection (hpi), and gradually increased as the infection progressed; no significant changes in PKR mRNA expression levels

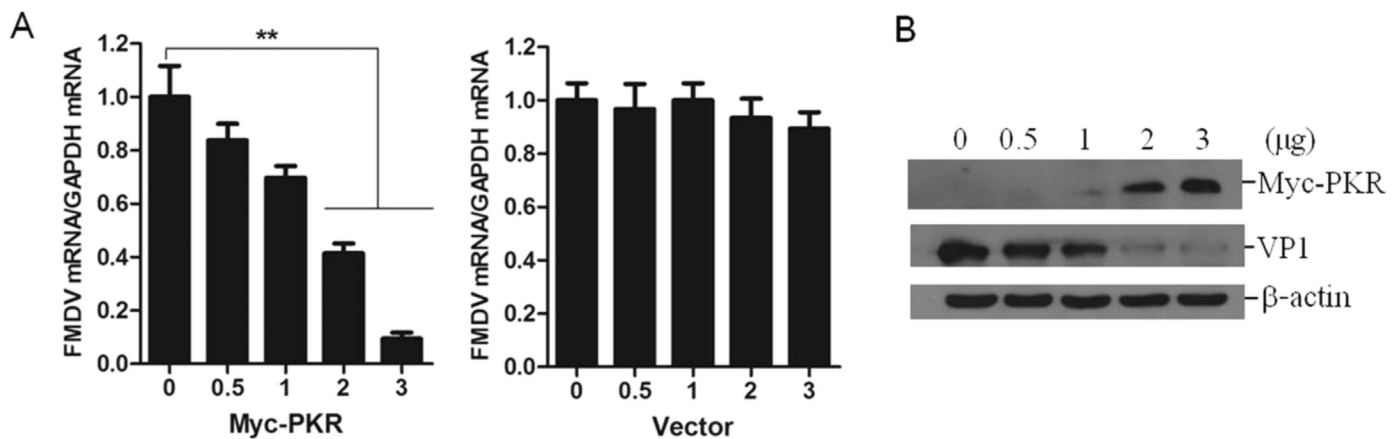
were observed in the mock-infected cells (Fig. 1A). To explore the correlation between PKR expression and FMDV replication, we also determined viral RNA levels. No viral RNA was detectable in mock-infected cells. In FMDV-infected cells, the viral RNA was detectable at 3 hpi, and gradually increased, similar to the increase of PKR (Fig. 1A). The state of PKR protein was also examined at different time points after FMDV infection, which showed that FMDV infection resulted in a significant loss of PKR protein. As shown in Fig. 1B, the amounts of PKR began to decrease at 6 hpi; and by 18 hpi, the amount of PKR almost could not be detected by western blotting. These results suggested that FMDV infection triggers PKR mRNA expression, while PKR protein levels are gradually reduced as infection progresses.

### 2.2. Upregulation of PKR inhibits FMDV replication

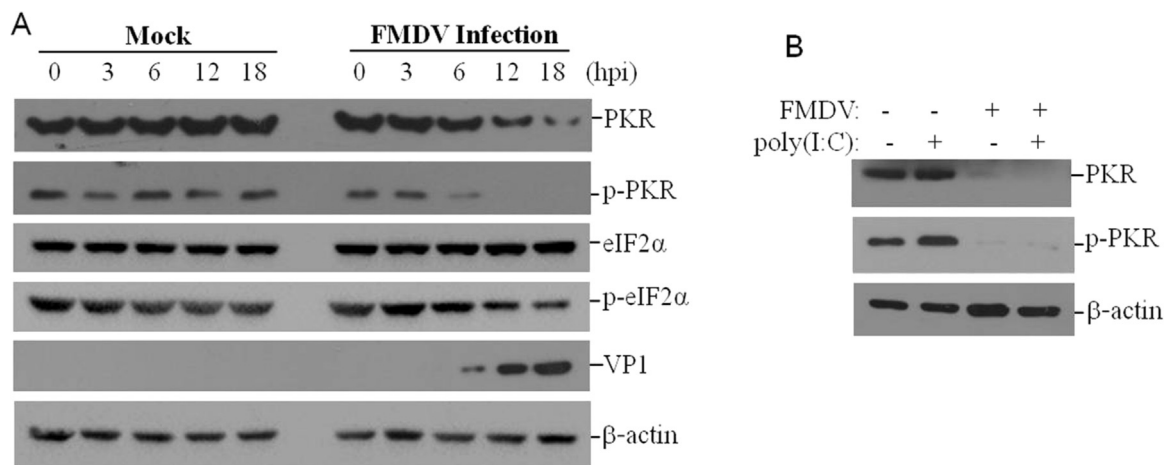
PKR plays an important role in inhibition of FMDV replication. FMDV infection reduced PKR expression. To explore whether upregulation of PKR suppresses FMDV replication, PK-15 cells were transfected with different amounts of empty vector plasmids or Myc-PKR expressing plasmids; at 12 h post-transfection (hpt), the cells were infected with 0.5 MOI of FMDV for 12 h. The viral RNA expression levels were determined and compared. The relative abundance of viral RNA was significantly reduced by overexpression of PKR, showing a dose-dependent manner (Fig. 2A). No remarkable inhibitive effect was observed in the vector-transfected cells. This observation was further confirmed by western blotting analysis. The presence of VP1 was used as an indicator of FMDV replication. As shown in Fig. 2B, a remarkable decrease of VP1 levels was detected in PKR overexpressing cells, and it also showed a clear dose-dependent manner. These results determined that overexpression of PKR significantly suppresses FMDV infection in the virus-infected cells, which indicated that the presence of PKR in FMDV-infected cells is essential for suppressing virus replication.



**Fig. 1.** FMDV infection inhibits PKR protein expression. (A) PK-15 cells were mock-infected or infected with FMDV for 0, 3, 6, 12 or 18 h, the transcripts of FMDV and PKR were detected by qPCR. (B) The expression levels of viral and PKR proteins in PK-15 cells were detected by western blotting at the indicated time points post-infection.



**Fig. 2.** High abundance of PKR inhibits FMDV replication. Upregulation of PKR suppresses FMDV replication in a dose-dependent manner in PK-15 cells. (A) PK-15 cells were transfected with increasing amounts of Myc-PKR or empty vector plasmids (0, 0.5, 1, 2 or 3  $\mu$ g). The cells were infected with equal amounts of FMDV (0.5 MOI) at 12 hpt for 12 h. The expression of viral RNA was detected by qPCR; and (B) The expression of viral protein VP1 was detected by western blotting.  $**P < 0.01$ .



**Fig. 3.** FMDV inhibits PKR activation. (A) PK-15 cells were mock-infected or infected with FMDV (0.5 MOI) for 0, 3, 6, 12 or 18 h. The expression levels of PKR, p-PKR, eIF-2 $\alpha$  and p-eIF-2 $\alpha$  levels were detected by western blotting. (B) PK-15 cells were transfected with 1  $\mu$ g of empty vector or poly(I:C); and the cells were mock-infected or infected with FMDV (0.5 MOI) for 12 h. The expression levels of PKR and p-PKR levels were detected by western blotting.

### 2.3. FMDV infection inhibits PKR activation

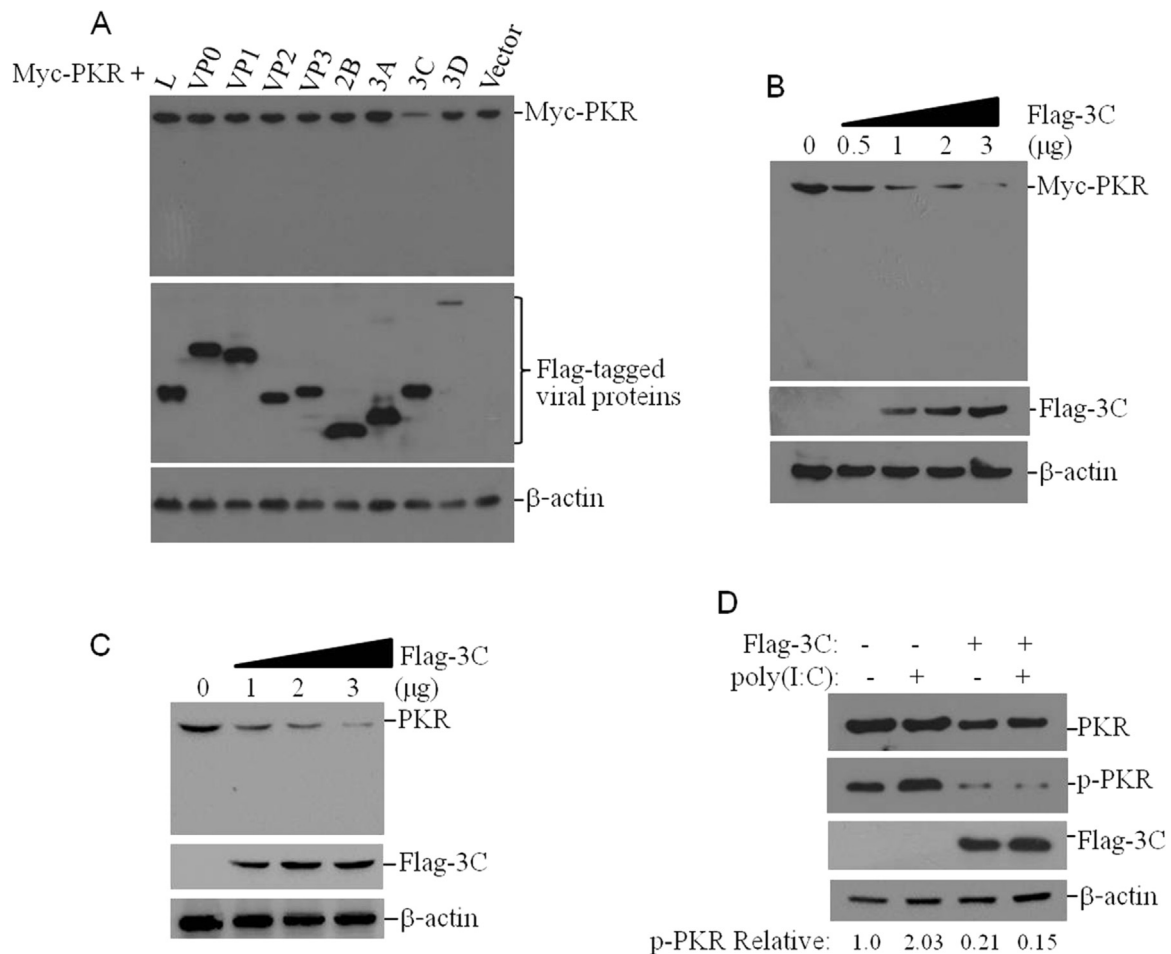
Poliovirus infection degrades PKR levels, while, the levels of phosphorylated PKR (p-PKR) significantly increased (Black et al., 1989). It suggests the decrease of total PKR levels not always results in a similar decrease of PKR phosphorylation levels. Therefore, we also investigated the state of PKR phosphorylation in FMDV-infected cells. The PK-15 cells were inoculated with 0.5 MOI of FMDV and harvested at 0, 3, 6, 12 or 18 hpi. Western blotting result showed that the level of p-PKR in FMDV-infected cells is significantly lower than that in the mock-infected cells (Fig. 3A). To further confirm this observation, we also investigated the effect of FMDV infection on poly(I:C)-induced PKR phosphorylation. poly(I:C) is an artificial dsRNA that can significantly induce the activation of PKR. PK-15 cells were transfected with poly(I:C) for 6 h, and then infected with FMDV for 12 h, the p-PKR levels were then assessed by western blotting. Transfection of poly(I:C) significantly induced PKR phosphorylation in the mock-infected cells. However, in the FMDV-infected cells, PKR phosphorylation was significantly blocked (Fig. 3B). The phosphorylation of the PKR substrate eukaryotic translation initiation factor 2 $\alpha$  (eIF-2 $\alpha$ ) in the FMDV-infected cells was also evaluated; the relative levels of the phosphorylated eIF-2 $\alpha$  (p-eIF2 $\alpha$ ) were also reduced after FMDV infection, and the total eIF2 $\alpha$  level had minimum change (Fig. 3A). These results indicate that FMDV infection blocks PKR activation.

### 2.4. 3C<sup>pro</sup> inhibits PKR protein expression and PKR activation

FMDV infection reduces PKR protein levels. To identify the viral proteins that are responsible for the decrease of PKR protein levels, various Flag-tagged viral proteins and Myc-tagged PKR expressing plasmids were co-transfected into HEK293T cells. The expression levels of Myc-PKR were measured by western blotting at 36 hpt. It was observed that 3C<sup>pro</sup> significantly reduced PKR abundance (Fig. 4A). The dose-dependent experiment was further performed, and the results confirmed 3C<sup>pro</sup>-mediated suppressive effect on PKR expression (Fig. 4B). 3C<sup>pro</sup>-induced decrease of endogenous PKR was also assessed by western blotting, and it showed that 3C<sup>pro</sup> significantly reduced endogenous PKR protein levels in a dose-dependent manner (Fig. 4C), which was consistent with those observed above. The effect of 3C<sup>pro</sup> on poly(I:C)-induced PKR phosphorylation was subsequently examined by Western blotting. It showed that 3C<sup>pro</sup> also significantly suppressed poly(I:C)-induced PKR phosphorylation (Fig. 4D). These results suggest FMDV 3C<sup>pro</sup> reduces PKR protein levels and suppresses PKR activation during FMDV infection.

### 2.5. 3C<sup>pro</sup>-induced PKR reduction is independent of disruption of eIF4G

The abundance of PKR mRNA in the Flag-3C-transfected cells was subsequently evaluated to investigate if the reduction of PKR

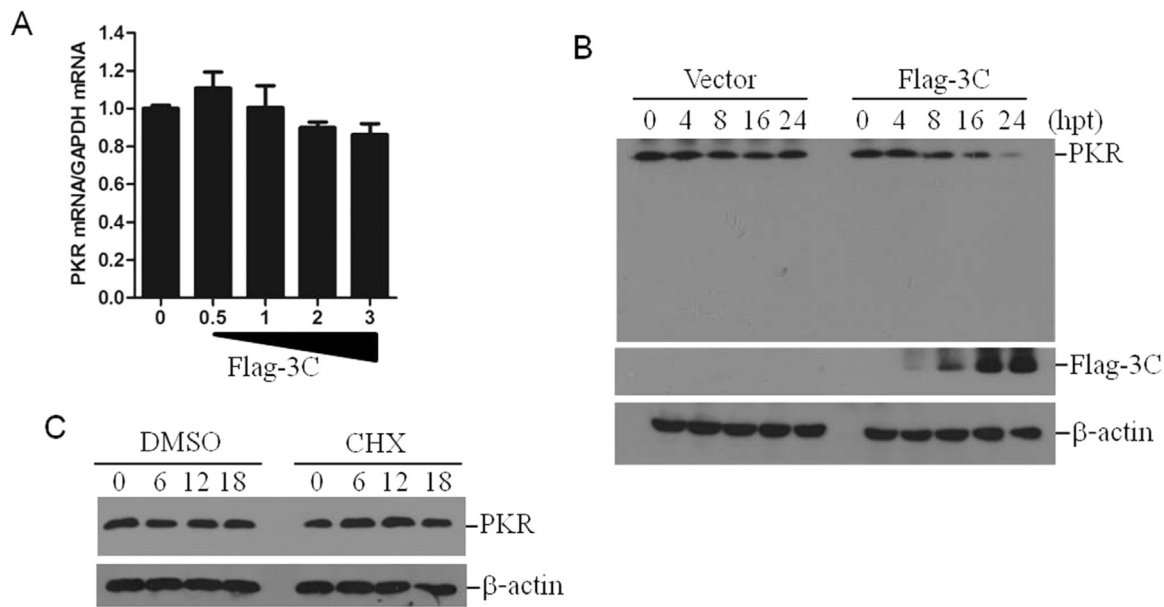


**Fig. 4.** 3C<sup>pro</sup> inhibits PKR protein expression and PKR activation. (A) HEK293T cells were co-transfected with 2  $\mu$ g Myc-PKR plasmids and 2  $\mu$ g vector plasmids or 2  $\mu$ g various Flag-tagged viral proteins expressing plasmids. The cells were collected at 36 hpt. The expressions of Myc-PKR and Flag-tagged viral proteins were detected by western blotting. (B) HEK293T cells were co-transfected with 2  $\mu$ g Myc-PKR plasmids and increasing amounts of Flag-3C plasmids (0, 0.5, 1, 2 or 3  $\mu$ g) for 36 h. The expressions of Myc-PKR were detected by western blotting. The empty vector plasmids were used in the transfection process to ensure that the same amount of cells received the same amount of total plasmids. (C) PK-15 cells were transfected with increasing amounts of Flag-3C plasmids (0, 1, 2 or 3  $\mu$ g) for 36 h. The empty vector plasmids were used in the transfection process to ensure that the same amount of cells received the same amount of total plasmids. The expressions of endogenous PKR were detected by western blotting. (D) PK-15 cells were transfected with vector plasmids or Flag-3C plasmids in the presence or absence of poly(I:C) (1  $\mu$ g). The PKR and p-PKR levels were detected by western blotting. Relative level of p-PKR is indicated in folds below the images after normalization with  $\beta$ -actin.

protein was the result of the specific decrease in mRNA expression. The expression of 3C<sup>pro</sup> had no significant affection on PKR mRNA expression (Fig. 5A). This indicates that 3C<sup>pro</sup>-induced PKR reduction was not due to reduced synthesis of PKR mRNA. 3C<sup>pro</sup> is a viral proteinase that can directly induce the cleavage of various host proteins. The kinetics of 3C<sup>pro</sup>-induced reduction of PKR were subsequently examined to detect any PKR cleavage product(s). As shown in Fig. 5B, 3C<sup>pro</sup> gradually induced the decrease of PKR as time progressed; however, no cleavage bands were observed. This suggests that 3C<sup>pro</sup> does not induce the cleavage of PKR. It is well known that 3C<sup>pro</sup> induces the cleavage of the eukaryotic cellular translation initiation factor 4G (eIF4G) and results in a so-called “shut-off” of host cell protein synthesis. To determine whether the reduction of PKR was completely due to this shut-off effect, a translational inhibitor cycloheximide (CHX) was used to evaluate this correlation. The PK-15 cells were mock-treated or treated with CHX for 0, 6, 12 or 18 h, the PKR levels were detected by western blotting. The inhibition of host protein synthesis did not result in detectable reduction of PKR for 18 h (Fig. 5C). This indicates that FMDV- or 3C<sup>pro</sup>-induced reduction of PKR protein levels is not related to the well-characterized blocking effect of 3C<sup>pro</sup> on cellular translation (viral host cell shutoff).

#### 2.6. 3C<sup>pro</sup> does not interact with PKR and the catalytic triad active-site residues of 3C<sup>pro</sup> are not essential for 3C<sup>pro</sup>-induced PKR reduction

To investigate whether there is a direct interaction between 3C<sup>pro</sup> and PKR, the co-immunoprecipitation assay was performed. HEK293T cells were co-transfected Myc-PKR plasmid and vector, Flag-L or Flag-3C plasmids. L<sup>pro</sup> showed no effect on PKR expression (Fig. 4), therefore, was used as a control. The cells were collected at 36 hpt, and the cell lysates were immunoprecipitated with anti-Myc antibody. As shown in Fig. 6A, PKR did not precipitate 3C<sup>pro</sup> or L<sup>pro</sup>. A reverse immunoprecipitation was also performed using Flag antibody, which showed that 3C<sup>pro</sup> or L<sup>pro</sup> did not precipitate PKR (Fig. 6B). These results suggested that there was no direct interaction between PKR and 3C<sup>pro</sup>. The catalytic triad of H46, D84, and C163 of FMDV 3C<sup>pro</sup> have been determined as crucial sites that were essential for the enzyme activity of 3C<sup>pro</sup> (Birtley et al., 2005; Du et al., 2014b; Grubman et al., 1995). To determine whether the enzyme activity was involved in 3C<sup>pro</sup>-induced PKR reduction, we constructed 3 mutant plasmids that expressing 3C<sup>pro</sup> mutant (Flag-3C-H46Y, Flag-3C-D84N and Flag-3C-C163G) which eliminated the protease activity of 3C<sup>pro</sup>. Another mutant plasmid Flag-3C-H205R that had no influence on the enzyme activity of 3C<sup>pro</sup> was constructed and used as a control. We co-



**Fig. 5.** 3C<sup>pro</sup> induces PKR reduction at posttranslational levels. (A) PK-15 cells were transfected with increasing amounts of Flag-3C plasmids (0, 0.5, 1, 2 or 3 μg) for 36 h. The empty vector plasmids were used in the transfection process to ensure that the same amount of cells received the same amount of total plasmids. The expressions of PKR mRNA levels were detected by qPCR method. (B) PK-15 cells were transfected with vector plasmids or Flag-3C plasmids. The cells were collected at 0, 4, 8, 16 or 24 hpt and subjected to western blotting analysis. The PKR protein levels were then detected and compared. (C) PK-15 cells were treated with DMSO (solvent control) or CHX (100 μg/mL) for 0, 6, 12 or 18 h, the expression of PKR was detected by western blotting.

transfected the vector plasmids, mutant plasmids or Flag-3C plasmids with Myc-PKR plasmids into the HEK293T cells, the expression levels of Myc-PKR were detected at 36 hpt. As shown in Fig. 6C, compared to the vector control, the wild-type 3C<sup>pro</sup>, 3C-H205R, 3C-H46Y, 3C-D84N and 3C-C163G all significantly repressed PKR expression. The cleavage of NEMO was used to evaluate the enzyme activity of 3C<sup>pro</sup> and 3C<sup>pro</sup> mutant. As expected, the mutation of H46Y, D84N and C163G indeed abolished the enzyme activity of 3C<sup>pro</sup> (Fig. 6C). It indicated that the elimination of the protease activity of 3C<sup>pro</sup> did not impair 3C<sup>pro</sup>-induced reduction of PKR.

### 2.7. 3C<sup>pro</sup> induces PKR reduction through lysosomal pathway

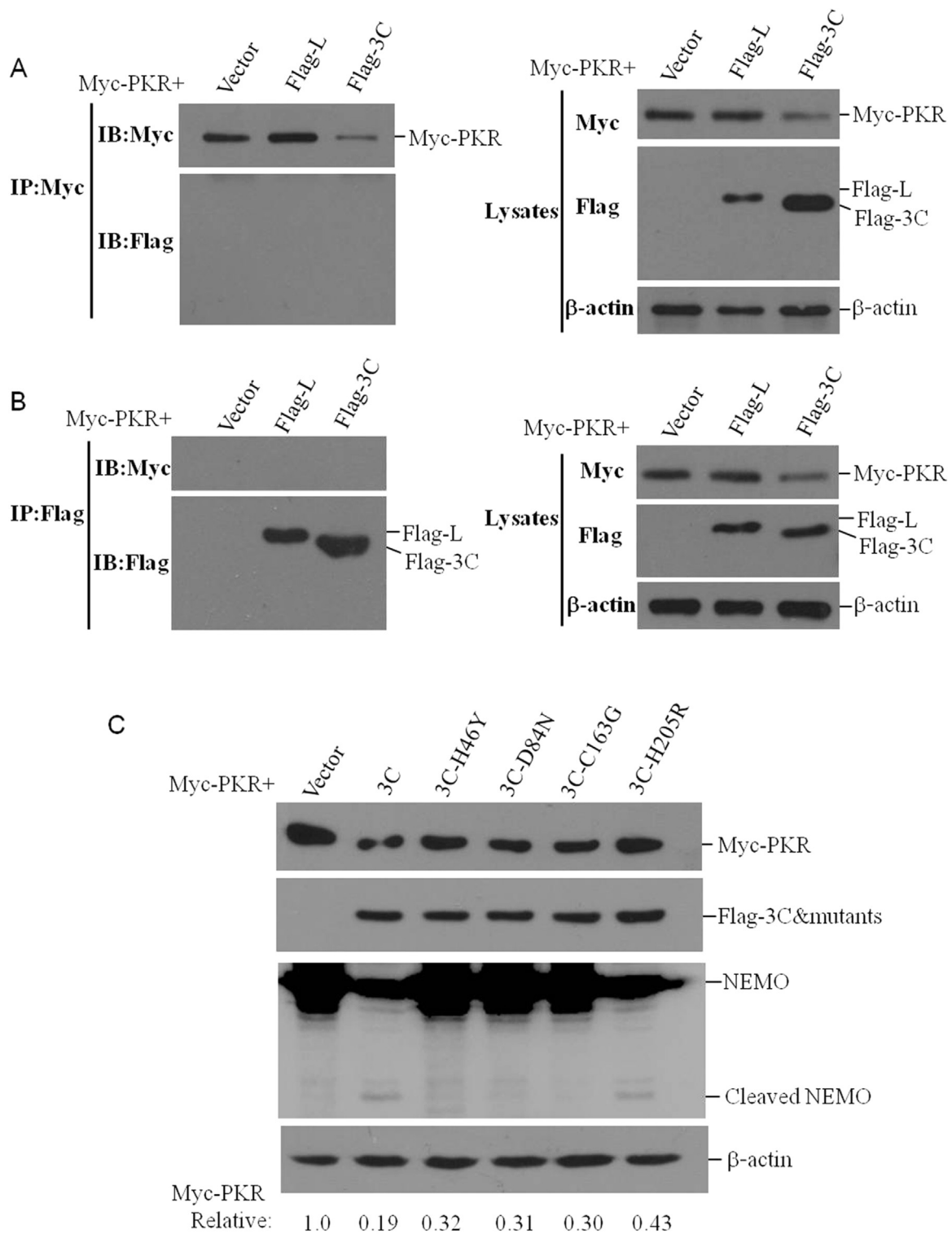
To investigate whether the proteasomes, lysosomes or caspase-dependent pathways are involved in FMDV- and 3C<sup>pro</sup>-induced decrease of PKR, the proteasome inhibitor MG132, lysosome inhibitor ammonium chloride (NH<sub>4</sub>Cl) and chloroquine diphosphate (CQ), and a general caspases inhibitor Z-VAD-FMK were used to examine the inhibitive effects. As show in Fig. 7A, incubation of CQ and NH<sub>4</sub>Cl significantly restored PKR levels in FMDV-infected cells. However, MG132 and Z-VAD-FMK had no such effect on PKR restoration. The effect of these inhibitors on 3C<sup>pro</sup>-induced decrease of PKR was also evaluated by western blotting, which indicated that incubation of CQ and NH<sub>4</sub>Cl also restored PKR levels in Flag-3C-transfected cells, and MG132 and Z-VAD-FMK had no such effect (Fig. 7B). Taken together, these results suggest that FMDV 3C<sup>pro</sup> induces PKR degradation through the lysosome pathway.

## 3. Discussion

FMDV is one of the most concerned animal virus due to its enormous potential to cause epidemics and economic losses (Kitching, 2005). FMDV infection strongly suppresses host innate antiviral responses by various strategies (Liu et al., 2015). 3C<sup>pro</sup>, as an important viral proteinase, is responsible for inducing cleavage of several viral polyprotein and has the ability to antagonize host antiviral effects (Curry et al., 2007). For instance, 3C<sup>pro</sup> specifically incises host translation initiation factor eIF4G, resulting in shut-off of host proteins

synthesis (Belsham et al., 2000). 3C<sup>pro</sup> also induces the nuclear factor kappa B (NF-κB) essential modulator (NEMO) and karyopherin α1 cleavage to impair innate antiviral response (Du et al., 2014b; Wang et al., 2012a). PKR, as a viral RNA sensor, plays important roles in host antiviral response (Balachandran et al., 2000; Dabo and Meurs, 2012). It has previously been shown that incubation of a PKR inhibitor, 2-AP, significantly increases the yield of FMDV in PK and EBK cells, suggesting an important antiviral role of PKR against FMDV (Chinsangaram et al., 2001). Our data here demonstrate that over-expression of PKR also suppresses FMDV, and FMDV infection degrades PKR to abate the antiviral action of PKR. This indicates that PKR functions as an antiviral factor during FMDV infection, and the virus has developed strategies to antagonize PKR-mediated antiviral action.

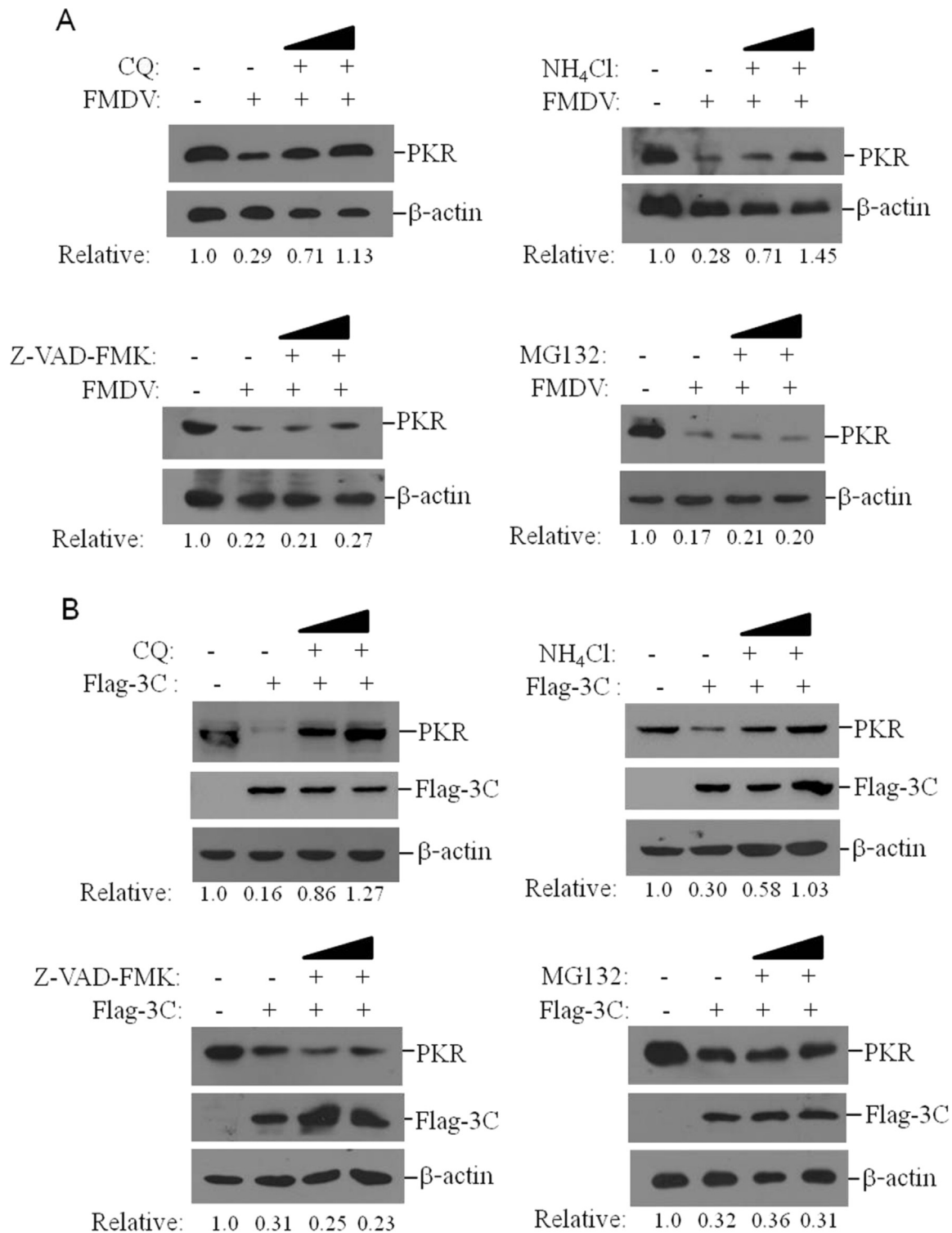
The importance of PKR in antiviral defense has been supported by various viruses, and PKR regulates diverse cellular pathways to perform its antiviral action (Balachandran et al., 2000; Garcia et al., 2006, 2007; Nakamura et al., 2010). Meanwhile, most viruses have developed mechanisms to block or impair PKR-induced antiviral response by using delicate strategies; the well-known strategies include expression of dsRNA-binding proteins or small RNAs which compete with PKR for its ligand, interaction with PKR by viral proteins, expression of a decoy substrate, or dephosphorylation of eIF2α (Garcia et al., 2007; Habjan et al., 2009; Langland et al., 2006; Lussignol et al., 2013; Xiao et al., 2016). For example, the accessory protein 4a of Middle East respiratory coronavirus prevents PKR activation by sequestering its ligand, dsRNA (Rabouw et al., 2016). NS5A protein of HCV directly interacts with PKR to inhibit PKR dimerization and activation (Tan et al., 1998). To our knowledge, rift valley fever virus (RVFV), Mengovirus and poliovirus are the only identified viruses that possess the activity to degrade PKR. NSs protein of RVFV specifically induces PKR degradation via the proteasome pathway (Habjan et al., 2009); the responsible viral factor for Mengovirus- and poliovirus-induced PKR degradation has not been clarified (Black et al., 1989; Feng et al., 2012). We report here that FMDV infection induces PKR degradation and 3C<sup>pro</sup> is the antagonistic factor responsible for this degradation. 3C<sup>pro</sup>-induced cleavage of eIF4G blocks the expression of many host proteins (Belsham et al., 2000). However, we found that 3C<sup>pro</sup> specifically reduces PKR protein



**Fig. 6.** 3C<sup>PRO</sup> does not interact with PKR and the protease activity is not essential for 3C<sup>PRO</sup> to induce PKR reduction. (A) HEK293T cells grown in the 10-cm dish were co-transfected with Myc-PKR (5 μg) and vector, Flag-L or Flag-3C plasmids (5 μg) for 36 h. The cell lysates were immunoprecipitated with mouse anti-Myc antibody and subjected to western blotting. (B) HEK293T cells grown in the 10-cm dish were co-transfected with Myc-PKR (5 μg) and vector, Flag-L or Flag-3C plasmids (5 μg) for 36 h. The cell lysates were immunoprecipitated with rabbit anti-Flag antibody and subjected to western blotting. (C) HEK293T cells were co-transfected with 2 μg of Myc-PKR plasmids and 3 μg of vector plasmids or various 3 C mutant plasmids for 36 h. The expression of Myc-PKR and host protein NEMO was detected by western blotting. Relative level of Myc-PKR was indicated in folds below the images after normalization with β-actin.

levels after inhibition of cellular translational function, indicating that 3C<sup>PRO</sup>-induced reduction of PKR protein levels is not totally related to the well-characterized blocking effect of 3C<sup>PRO</sup> on cellular translation. L<sup>PRO</sup> also induces the cleavage of host eIF4G to inhibit host protein synthesis (Kirchweger et al., 1994); however, we observed that over-

expression of L<sup>PRO</sup> had no detectable effect on PKR expression (Fig. 4A); it was similar to the observation in the cells treated with a translational inhibitor CHX (Fig. 5C), and the long half-life of PKR may result in this minimum change. This also explains why L<sup>PRO</sup> does not degrade PKR. The co-transfection of the plasmids expressing other FMDV proteins



**Fig. 7.** FMDV and 3C<sup>pro</sup> induces PKR reduction through lysosomal pathway. (A) PK-15 cells were mock-infected or infected with FMDV (0.5 MOI) in the absence or presence of CQ (50 or 100  $\mu$ M), NH<sub>4</sub>Cl (10 or 20 mM), Z-VAD-FMK (10 or 50  $\mu$ M) or MG132 (2 or 20  $\mu$ M). The expression of PKR was detected by western blotting at 12 hpi. (B) PK-15 cells were transfected with 2  $\mu$ g vector plasmids or 2  $\mu$ g Flag-3C plasmids in the absence or presence of CQ (50 or 100  $\mu$ M), NH<sub>4</sub>Cl (10 or 20 mM), Z-VAD-FMK (10 or 50  $\mu$ M) or MG132 (2 or 20  $\mu$ M). The expression of PKR was detected by western blotting at 36 hpi. Relative level of PKR was indicated in folds below the images after normalization with  $\beta$ -actin.

with the PKR expressing plasmids showed no significant affection on PKR protein levels. These results suggest that 3C<sup>pro</sup> performs an important role in induction of PKR decrease in FMDV-infected cells.

Mengovirus infection induces significant decrease of total PKR protein levels (Dubois and Hovanessian, 1990). A recombinant mengovirus virus (mengovirus-Zn) which includes mutations in the Zn finger domain of the leader protein causes the decrease of total PKR levels

similar as the wildtype Mengovirus in the virus-infected cells. However, the level of PKR phosphorylation in the mengovirus-Zn-infected cells is strongly induced even with reduced PKR levels (Rabouh et al., 2016). Poliovirus infection also triggers PKR phosphorylation, even though PKR is significantly degraded (Black et al., 1989). These indicate that the PKR phosphorylation status and PKR abundance are not always correlated with each other. Our data reveal that both FMDV infection



and transfection of 3C<sup>pro</sup> clearly suppress the PKR phosphorylation induced by poly(I:C). The state of PKR phosphorylation and PKR levels are similar during FMDV infection or transfection of 3C<sup>pro</sup>. Therefore, we deem that the degradation of PKR may contribute to the decreased PKR phosphorylation in FMDV-infected cells.

Both L<sup>pro</sup> and 3C<sup>pro</sup> possess the proteinase activity, we observed that PKR could not be cleaved during FMDV infection or transfection of L<sup>pro</sup> or 3C<sup>pro</sup> (Fig. 1C and Fig. 4). It indicates that PKR degradation by FMDV 3C<sup>pro</sup> occurs in a specific manner and not by the proteinase activity. The mutation of the catalytic sites of 3C<sup>pro</sup> further confirmed this characteristic. No direct interaction was identified between PKR and 3C<sup>pro</sup>, the lysosomal pathway was determined to be involved in 3C<sup>pro</sup>-induced PKR reduction. Many RNA viruses induce degradation of host cell proteins to facilitate virus replication. Such as, in influenza A virus infection, the viral nonstructural protein NS1 induces lysosomal degradation of eukaryotic translation initiation factor 4B to promote virus replication (Wang et al., 2014); HIV Nef protein promotes lysosomal targeting of host CD4 receptor to facilitate release of newly produced viral particles (Amorim et al., 2014). How 3C<sup>pro</sup> induces PKR degradation in the lysosomes without a direct interaction remains unknown. Experiments to further characterize the degradation mechanism are currently under way.

In conclusion, our study demonstrated the significant antiviral role of PKR during FMDV infection. It also showed a novel mechanism by which FMDV 3C<sup>pro</sup> has evolved to induce a degradation of PKR by lysosomal pathway and counteract PKR-induced antiviral effect.

## 4. Materials and methods

### 4.1. Antibodies

The commercial antibodies against PKR, Flag and  $\beta$ -Actin were purchased from Sigma-Aldrich. Rabbit anti-phospho-PKR (Thr451) antibody was purchased from Millipore Corporation (Merck Millipore). Mouse anti-eIF2 $\alpha$  monoclonal antibody and rabbit anti-phospho-eIF2 $\alpha$  (Ser51) (D9G8) XP were purchased from Cell Signaling Technology (CST). Mouse anti-myc monoclonal antibody and HRP-conjugated goat anti-rabbit IgG antibody, as well as HRP-conjugated goat anti-mouse IgG antibody were purchased from Santa Cruz Biotechnology. An anti-VP1 polyclonal antibody was generated in our laboratory (unpublished data).

### 4.2. Viruses and cells

Porcine kidney (PK-15) cells and human embryonic kidney 293 T (HEK293T) cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's Modified Eagle Media (DMEM, Invitrogen), supplemented with 10% heated-inactivated fetal bovine serum (FBS) and then cultured at 37 °C in a humidified 5% CO<sub>2</sub> incubator. FMDV strain O/BY/CHA/2010 (GenBank number: JN998085) was conserved by National Foot and Mouth Diseases Reference Laboratory, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences and used for virus infection in this study.

### 4.3. Plasmid constructions and transfection

A Myc-tagged PKR eukaryotic expression construct was generated through inserting full-length porcine PKR cDNA fragment into a pcDNA3.1/myc-His A vector. Various pCAGGs plasmids expressing Flag-tagged viral protein were constructed from the cDNA of FMDV strain O/BY/CHA/2010 by standard reverse transcription (RT)-PCR by our lab previously as described (Li et al., 2016a). Specific mutations (H46Y, D84N, C163G, and H205R) were introduced to pCAGGs-3C by the site-directed mutagenesis PCR as previously described (Zhu et al.,

2013). All the plasmids were verified by DNA sequencing analysis. The plasmids were transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

### 4.4. Viral infection

The viral infection experiment was carried out as previously described (Li et al., 2016b). Briefly, the cell cultures were washed with PBS for 3 times, and the virus was incubated for 1 h (h) in serum-free medium at the multiplicity of infection (MOI) of 0.5. The infected cells were then washed with PBS, and the supernatant was removed and then maintained in fresh medium supplemented with 1% FBS. The mock infection was carried out as the viral infection method using the serum-free medium instead of the virus for incubation. The samples were collected at different hours post-infection (hpi).

### 4.5. RNA extraction, reverse transcription, and real-time PCR

Total RNAs were isolated from the cells using the TRIzol<sup>®</sup> Reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription (RT)-PCR was conducted to synthesize cDNAs. M-MLV reverse transcriptase (Promega) and random hexamer primers (TaKaRa) was used for cDNA amplification in RT-PCR. The generated cDNAs were used for quantitative real-time PCR (qPCR) assay. The cDNAs were quantified using SYBR Premix Ex Taq reagents (TaKaRa) and the Mx3005 P QPCR System (Agilent Technologies). Detection of house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also conducted for normalization of total input RNA. Relative transcript levels were calculated by  $2^{-\Delta\Delta CT}$  (where CT is threshold cycle) method as previously described (Zhu et al., 2016). The primers used in this study were shown in Table 1.

### 4.6. Western blotting

The collected cell samples were lysed in the lysis buffer supplemented with phenylmethylsulfonyl fluoride (PMSF; Sigma). The protein-containing supernatants were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P membranes (Millipore). 5% skim milk in TBST was used to block the membranes. The membranes were incubated and probed with appropriate antibodies to generate antibody-antigen complexes. The generated complexes were visualized by enhanced chemiluminescence detection reagents (Thermo). Beta-actin was determined as a protein loading control.

### 4.7. Chemical inhibitors assay

The proteasome inhibitor MG132, the lysosome inhibitor chloroquine diphosphate (CQ) and NH<sub>4</sub>Cl, and caspase inhibitor benzoyloxycarbonyl (Cbz)-L-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-FMK) were used to suppress various pathways involved in the protein degradation process. Monolayer PK-15 cells were mock-infected or infected with FMDV (0.5 MOI) in the absence or presence of CQ (50  $\mu$ M or 100  $\mu$ M), NH<sub>4</sub>Cl (10 mM or 20 mM), Z-VAD-FMK (10  $\mu$ M or 50  $\mu$ M) or MG132 (2  $\mu$ M or 20  $\mu$ M); the cells were collected at 12 hpi, and then subjected to western blotting analysis. As for transfection of Flag-3C expressing plasmids, the monolayer cells were transfected with 2  $\mu$ g of Flag-3C plasmids or empty vector using Lipofectamine 3000 and maintained in the absence or presence of CQ (50  $\mu$ M or 100  $\mu$ M), NH<sub>4</sub>Cl (10 mM or 20 mM), Z-VAD-FMK (10  $\mu$ M or 50  $\mu$ M) or MG132 (2  $\mu$ M or 20  $\mu$ M) for 36 h. The expression levels of PKR and 3C<sup>pro</sup> were then detected by western blotting. The cycloheximide (CHX) was used to block protein synthesis of cells and evaluate the half-life of PKR protein. The PK-15 cells were cultured in the presence or absence of 100  $\mu$ g/mL of CHX for the indicated periods at 37 °C (Wang et al., 2012b). The collected cells were subjected to western blotting analysis.

**Table 1**

The primers used in this study.

Gene	Primers (5'→3')	Purpose
PKR	Forward: GGAAGAAAACAAACACAGCTTGAA Reverse: CCAATCCACCTGAGCCAATT	qPCR
GAPDH	Forward: ACATGGCCTCCAAGGAGTAAGA Reverse: GATCGAGTTGGGGCTGTGACT	qPCR
FMDV	Forward: CACTGGTGACAGGCTAAGG Reverse: CCCTTCTCAGATTCGGAGT	qPCR
PKR	Forward: ATCTCGAGATGGCCAGTGGTTCACCGTGT Reverse: CGTGGTACCACATGTGTTTCGTTCTTTTCTCTGTGACG	CDS amplification
3C-H46Y	Forward: TACCTCGTGCCCTGTTATCTCTTCGCAGAGAAG Reverse: CTTCTCTGCGAAGAGATAACGAGGCACGAGGTA	3C mutagenesis
3C-D84N3C-D84N	Forward: CAGGACATGCTCTCAAACGCCGCTCATGGTG Reverse: CACCATGAGCGCGGCTTTGAGAGCATGTCTCTG	3C mutagenesis
3C-C163G	Forward: ACCAAGGCTGGCTACGGTGGAGGAGCCGTTCTC Reverse: GAGAACGGCTCTCCACCGTAGCCAGCCTTGTT	3C mutagenesis
3C-H205R	Forward: CTTAAAATGAAGGCACGCATTGACCCCGAACCA Reverse: TGGTTCGGGGTCAATGCGTCCCTTCATTTTAAAG	3C mutagenesis

#### 4.8. Statistical analysis

All the experiments were repeated at least three times. The results were present as mean values  $\pm$  standard error (mean  $\pm$  SE) of three independent experiments. The Student's *t*-test was used to analyze the statistically significance (two-tailed Student's *t*-test). Statistical values of  $*P < 0.05$  was considered significant, and  $**P < 0.01$  was considered highly significant.

#### Conflicts of interest

The authors declare that they have no competing interests.

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