Biochemical and Biophysical Research Communications 399 (2010) 72–78

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Foot-and-mouth disease virus leader proteinase inhibits dsRNA-induced type I interferon transcription by decreasing interferon regulatory factor 3/7 in protein levels

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ARTICLE INFO

Article history:
Received 7 July 2010
Available online 16 July 2010

Keywords:
Foot-and-mouth disease virus (FMDV)
Leader proteinase
Type I interferon
Interferon regulatory factor

ABSTRACT

The leader proteinase (L^{pro}) of foot-and-mouth disease virus (FMDV) has been identified as an interferon-β (IFN-β) antagonist that disrupts the integrity of transcription factor nuclear factor κB (NF-κB). In this study, we showed that the reduction of double stranded RNA (dsRNA)-induced IFN-α1/β expression caused by L^{pro} was also associated with a decrease of interferon regulatory factor 3/7 (IRF-3/7) in protein levels, two critical transcription factors for activation of IFN-α/β. Furthermore, overexpression of L^{pro} significantly reduced the transcription of multiple IRF-responsive genes including 2′,5′-OAS, ISG54, IP-10, and RANTES. Screening L^{pro} mutants indicated that the ability to process eIF-4G of L^{pro} is not required for suppressing dsRNA-induced activation of the IFN-α1/β promoter and decreasing IRF-3/7 expression. Taken together, our results demonstrate that, in addition to disrupting NF-κB, L^{pro} also decreases IRF-3/7 expression to suppress dsRNA-induced type I IFN production, suggesting multiple strategies used by FMDV to counteract the immune response to viral infection.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of wild and domestic cloven-hoofed animals. FMD causes severe economic losses due to the decline of livestock production and international restrictions on exports of animals and animal products [1]. The etiologic agent, FMD virus (FMDV), is a positive-stranded RNA virus that belongs to the *Aphthovirus* genus of the *Picornaviridae* family. The genome of FMDV is a positive-strand RNA of 8.5 kb that is translated into a polyprotein. The polyprotein yields structural and nonstructural proteins upon cleavage by three virus-encoded proteinases, leader (L^{pro}), 2A, and $3C^{pro}$ [2]. L^{pro}, the first protein to be translated, is initiated at two different AUGs that are separated by 84 nucleotides, and results in two alternative forms of L^{pro}, termed Lab^{pro} and Lb^{pro}, respectively. Both forms have been detected in vitro and in infected cells [3,4].

The L^{pro} is a well-characterized papain-like proteinase [5,6] that self-cleaves from the nascent polyprotein precursor and also cleaves the host translation initiation factor eIF-4G, an event that results in the reduction of host cap-dependent mRNA translation [7,8]. Using a genetically engineered FMDV lacking the L^{pro} coding region (A12-LLV2), de Los Santos et al. [9] demonstrated that L^{pro} inhibits the induction of transcription of IFN-β mRNA and blocks the host innate immune response. These results indicate that L^{pro} is an antagonist of IFN-β. Further studies revealed that L^{pro} is associated with the degradation of p65/RelA, the subunit of nuclear factor κB (NF-κB), and, thus, affects the NF-κB activity required for the full expression of IFN-β and other inflammatory cytokines [10].

It is well known that IFN-β transcription requires the activation of transcription factors NF-κB, interferon regulatory factors (IRFs), and ATF2-c-Jun (AP-1) and their subsequent binding to the IFN-β enhancer [11]. In order to combat the antiviral effects of IFN-β, many viruses have evolved distinct strategies to inhibit IFN-β signaling pathways for their survival [12]. For example, influenza A virus NS1 prevents the potent antiviral interferon response by inhibiting IRF-3 activation [13], while African swine fever virus (ASFV) encodes an IκB orthologue that inhibits the activity of NF-κB to prevent IFN-β production [14]. Rotavirus nonstructural protein 1 (NSP1) represses the IFN response not only by inhibiting activation of NF-κB [15], but also by inducing proteasome-dependent degradation of IRF-3, IRF-5, and IRF-7 [16]. To fully understand the molecular mechanisms underlying FMDV L^{pro} inhibition of IFN-β expression, we here investigated whether L^{pro}, in the absence of other FMDV proteins, can inhibit dsRNA-induced IFN-β expression. The results show that L^{pro} suppresses dsRNA-induced IFN-β mRNA transcription through decreasing IRF-3/7 expression. Furthermore, L^{pro} suppresses dsRNA-induced IFN-α1 mRNA transcription. In addition, we also demonstrate that the ability to process eIF-4G of L^{pro} is not required to suppress dsRNA-induced activation of the IFN-α1/β promoter and decrease IRF-3/7 expression.

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doi:10.1016/j.bbrc.2010.07.044

2. Materials and methods

2.1. Cells and virus

Baby hamster kidney (BHK-21) and porcine kindey (PK-15) cells were cultured and maintained in Dulbecco's Modified Eagle Media (DMEM, Invitrogen), supplemented with 10% heated-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 10 μg/mL streptomycin sulfate and then held at 37 °C in a humidified 5% CO_2 incubator. FMDV strain O/ES/2001 was propagated in BHK-21 cells and the supernatants of infected cells were clarified and stored at −80 °C.

2.2. Plasmid constructions

The luciferase reporter plasmids IFN-β-Luc, 4× PRDII-Luc, and 4× PRDIII/I-Luc were described previously [17]. IFN-α1-Luc expresses firefly luciferase under the control of the porcine IFN-α1 promoter (from −411 to +38, the +1 position refers to the transcriptional start site of Accession No. X57191). DNA expression constructs encoding porcine IFN-beta promoter stimulator 1 (IPS-1) has been described previously [17]. The DNA expression constructs encoding full-length MDA5 (melanoma-differentiation-associated gene 5), IRF-3, IRF-7 and p65 were generated by standard reverse transcription (RT)-PCR from total RNA extracted from porcine peripheral blood mononuclear cells (PBMCs) and cloned into pCMV-Tag 2B (Stratagene). The phosphomimetic mutation IRF-3(5D) was introduced into porcine IRF-3 by replacing Ser-394, Ser-396, Ser-400, Thr-402, and Ser-403 residues with the phosphomimetic aspartic acid. The mutations of a constitutively activated form IRF-3(5D) and a constitutively activated form IRF-7(Δ243–451) were generated in analogy to mutations described of human IRF-3/7 [18,19] and were subcloned into pCMV-Tag 2B (Stratagene).

For construction of pCMV-HA/L^{pro}, the DNA fragment containing the full-length Lab^{pro} and Lb^{pro} of FMDV were amplified by PCR from the cDNAs of FMDV O/ES/2001 (GenBank Accession No. AY686687) and subcloned into pCMV-HA vector (Clontech). Mutagenesis of amino acids C51A, I83A, L86A, D163N, and D164N in Lab^{pro} and Lb^{pro} were generated by using the Overlap Extension Polymerase Chain Reaction. The details of the specific primers used in PCR steps are available on request. All constructs was confirmed by sequencing.

2.3. Transfection and reporter gene assays

PK-15 cells were seeded in 24-well plates and incubated until the cells reached approximately 70–80% confluence. Cells were then co-transfected with 0.1 μg/well of a reporter plasmid along with 0.05 μg/well of pRL-TK plasmid (Promega) for normalization and other various expression plasmids or an empty control plasmid. In some selected experiments, 1 μg of poly(I:C) were transfected at 24 h after the initial co-transfection. Cells were harvested 12 h later and firefly and Renilla luciferase activities were determined using a dual-luciferase reporter assay system (Promega) according to the manufacturer's directions. Data represent relative firefly luciferase activity normalized to Renilla reniformis luciferase activity.

2.4. RNA exaction and quantitative real-time RT-PCR

To determine the effect of L^{pro} on the porcine IRF-3, IRF-7, 2′,5′-OAS (2′,5′-oligoadenylate synthetase), ISG54 (IFN stimulated gene 54), IP-10 (IFN-inducible protein 10, also known as CXCL10) and RANTES (regulated upon activation, normal T-cell expressed and secreted, also known as CCL5), PK-15 cells in 24-well plates were transfected with 1 μg of empty plasmid encoding Lab^{pro} or Lb^{pro}, and 24 h after the initial transfection, the cells were transfected with 1 μg of poly(I:C). Twelve hours later, RNAs were extracted from the cells by using TRIzol reagent (Invitrogen). Total RNAs (1 μg) were reverse transcribed to cDNAs using reverse transcriptase from TOYOBO, and 1 μl of the total cDNA volume was used in a SYBR green PCR assay (Applied Biosystems). Individual transcripts in each sample were assayed three times and normalized with porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level as an endogenous control. Primers were designed with Primer Express software v.3.0 (Applied Biosystems). The sequences for the primers are listed in Table S1.

2.5. Western blot analysis

Briefly, PK-15 cells cultured in 60 mm-dishes were co-transfected with pCMV-Tag/IRF-3, pCMV-Tag/IRF-7, or pCMV-Tag/p65

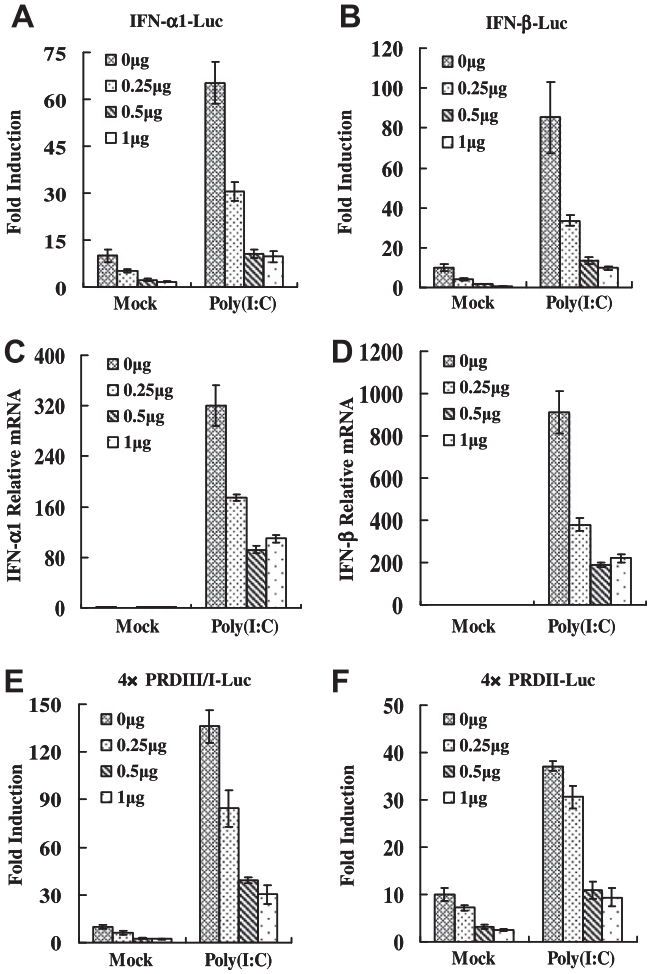

Fig. 1. FMDV L^{pro} independently inhibits IFN-α1/β promoter activation by disrupting activation of IRF-3/7 and NF-κB. (A,B) PK-15 cells were transfected with 0.1 μg/well of the reporter plasmid, IFN-α1-Luc, IFN-β-Luc, along with 0.05 μg/well of pRL-TK plasmid and increasing quantities (0, 0.25, 0.5, or 1 μg) of plasmid encoding Lab^{pro}, using Lipofectamine 2000. Twenty-four hours after the initial transfection, the cells were transfected with 1 μg of poly(I:C). Luciferase assays and real-time RT-PCR were performed at 12 h after the second transfection. Results represent the means and standard deviation of three independent experiments. The relative firefly luciferase activity was normalized to the Renilla reniformis luciferase, and the untreated empty vector control value was set to 10. (C,D) RNAs were extracted to measure porcine IFN-α1 (C) and IFN-β (D) mRNA by real-time RT-PCR. (E,F) The experiments were similarly performed as in Fig. 1A and B except that the 4× PRDIII/I-Luc (E), or 4× PRDII-Luc (F) promoter reporter plasmid was used.

plus indicated amounts of constructs containing L^{pro}. Empty vector pCMV-HA was used to standardize the total amount of DNA used for transfection. After 48 h, cells were harvested by adding lysis buffer, and protein concentration is measured and adjusted. The same amounts of each protein sample were then analyzed for IRF-3, IRF-7, or p65 proteins by Western blotting with anti-Flag M2 antibody (Sigma). To confirm the expression levels of L^{pro}, anti-HA antibody (Sigma) was used to detect the HA-tagged proteins. Beta-actin was detected with anti-beta-actin mAb antibody (Sigma) as a protein loading control.

2.6. Statistical analysis

All experiments were performed at least three times with reproducible results. Data are presented as means ± SD. Statistical values of $^{*}P < 0.05$ were considered significant and $^{**}P < 0.01$ highly significant.

3. Results and discussion

3.1. FMDV L^{pro} independently inhibits poly(I:C)-induced IFN-α1/β mRNA expression

By using a genetically engineered FMDV lacking the L^{pro} coding region, two general models of the IFN-antagonistic function of L^{pro} have been proposed in previous studies [10,20]. L^{pro} mainly works by degrading p65/RelA, the subunit of NF-κB [10]. Alternatively, L^{pro} blocks the expression of cellular genes at the post-transcriptional level and, thereby, IFN gene expression [20]. To investigate whether L^{pro}, in the absence of other FMDV proteins, can inhibit dsRNA-induced IFN-α/β expression, PK-15 cells were transfected with a HA-tagged Lab^{pro} expression construct (pHA-Lab), together with a luciferase reporter plasmid with the porcine IFN-α1 promoter or the IFN-β promoter and pRL-TK. At 24 h post-transfection, the cells were further transfected or mock-transfected with poly(I:C) followed by the dual-luciferase assay. To minimize exper-

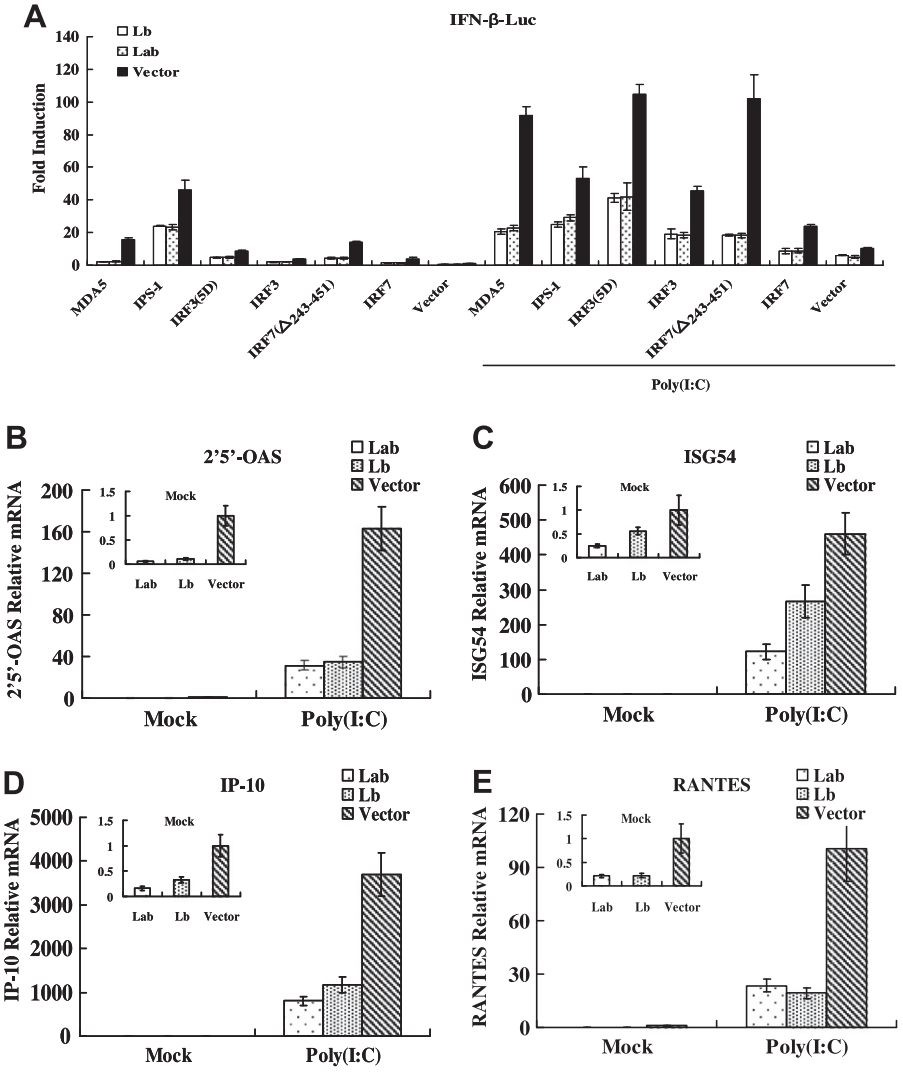

Fig. 2. L^{pro} significantly reduced the transcription of multiple IRF-responsive genes. (A) PK-15 cells were co-transfected with the IFN-β-Luc reporter plasmid (0.1 μg), pRL-TK plasmid (0.05 μg), and 0.5 μg of plasmid encoding Lab^{pro} or Lb^{pro} together with the MDA5, IPS-1, IRF-3, IRF-7, IRF-3(5D), or IRF-7(Δ243–451) expression vector (0.5 μg). Twenty-four hours after the initial transfection, the cells were transfected with 1 μg of poly(I:C). Luciferase assays were performed at 12 h after the second transfection. (B–E) PK-15 cells were transfected with 1 μg of plasmid encoding Lab^{pro}, Lb^{pro}, or an empty vector (pCMV-HA), and, 24 h later, the cells were transfected with 1 μg of poly(I:C). Twelve hours after the second transfection, total RNA was extracted and the expression of 2′,5′-OAS (B), ISG54 (C), IP-10 (D), RANTES (E), and GAPDH genes were evaluated by quantitative real-time RT-PCR. Results are expressed as increases in mRNA levels relative to those in cells transfected in the absence of poly(I:C) and were normalized by using GAPDH housekeeping gene expression. Results are representative of those from three independent experiments.

imental variability in the host cell translation caused by L^{pro}, the results of the reporter assay were normalized to the luciferase controls. As shown in Fig. 1A and B, Lab^{pro} also down-regulated the relative activity of the IFN-α1/β promoter, suggesting that Lab^{pro} may inhibit IFN-α1/β mRNA transcription. To support this hypothesis, the mRNA expression of IFN-α1 and IFN-β were further analyzed by real-time RT-PCR in parallel. The results showed that poly(I:C)-induced IFN-α1/β mRNA expression were significantly inhibited by Lab^{pro} (Fig. 1C and D). Similar results were observed in cells overexpressing Lb^{pro}, another alternative form of L^{pro} (data not shown). Taken together, these results suggest that L^{pro} is involved independently in the inhibition of IFN-α1/β transcription.

3.2. FMDV L^{pro} independently inhibits IFN-α1/β promoter activation by disrupting activation of IRF-3/7 and NF-κB

Transcription of the IFN-β gene is dependent on the activity of AP-1, NF-κB, and IRF-3/7, and the latter two are crucial for IFN transcription in response to viral infection [21]. Although the promoter of IFN-α contains several copies of IRFs binding sites, no AP-1 or NF-κB binding sites are present [22]. L^{pro} can significantly inhibit poly(I:C)-induced IFN-α1 promoter activation, suggesting that it can inhibit the activity of IRFs. To test this hypothesis, the luciferase reporter plasmids 4× PRDIII/I-Luc, which contain four copies of the IRF-3/7-binding positive regulatory domain (PRD) motif of the porcine IFN-β promoter in front of a firefly luciferase reporter gene, was co-transfected with pHA-Lab and luciferase reporter assays were performed. As shown in Fig. 1E, overexpression of Lab^{pro} significantly inhibited poly(I:C)-mediated IRF-3/7 activation in a dose-dependent manner. Furthermore, overexpression of Lab^{pro} also inhibited poly(I:C)-mediated NF-κB activation (Fig. 1F). Similar results were observed in Lb^{pro}-expressing cells (data not shown). These results indicate that, in addition to NF-κB, reduction of dsRNA-induced IFN-α/β expression caused by L^{pro} is associated with the inhibition of IRF-3/7. Similar conclusions have been reported in other *picornaviruses*. For example, the L^{pro} of Theiler's virus is associated with a block in the induction of IFN-α/β upon viral infection by blocking the translocation of IRF-3 to the nucleus [23,24]. In addition, the L^{pro} of Mengovirus has been demonstrated to block the induction of IFN-α/β by preventing both NF-κB and IRF-3 activity [25,26].

3.3. L^{pro} inhibits MDA5-mediated activation of the IFN-α1/β promoter

MDA5 has been shown to be crucial for sensing infections by picornaviruses [27]. Based on the proposed central role of RIG-I/MDA5 as a cellular sensor of viral products resulting in the activation of IRF-3/7 and induction of IFN-α/β and the fact that L^{pro} of

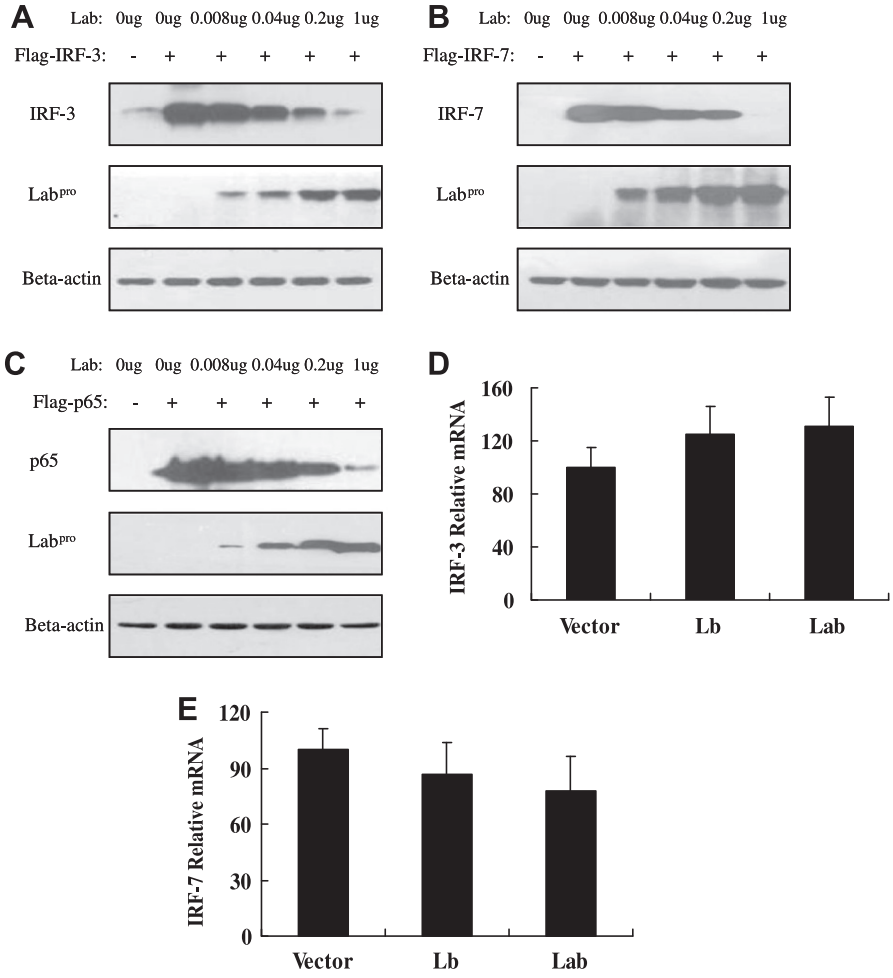

Fig. 3. L^{pro} decreases IRF-3/7 in protein levels. (A,C) PK-15 cells were transfected with IRF-3, IRF-7, or p65 (2), along with increasing quantities (0, 0.008, 0.04, 0.2, or 1 μg) of plasmid encoding Lab^{pro}, using Lipofectamine 2000. Cell lysates were prepared at 48 h post-transfection and analyzed for Flag-IRF-3/7 or p65 conjugated proteins by Western blot with an anti-Flag antibody. Mouse anti-HA was used to confirm the expression of Lab^{pro}, and anti-beta actin antibody was used to detect beta actin, which serves as a protein loading control. (D,E) PK-15 cells were transfected with 1 μg of plasmid encoding L^{pro} or an empty vector (pCMV-HA). Forty-eight hours after transfection, total RNA was extracted, and the expression of IRF-3/7 or GAPDH gene was evaluated by quantitative real-time RT-PCR.

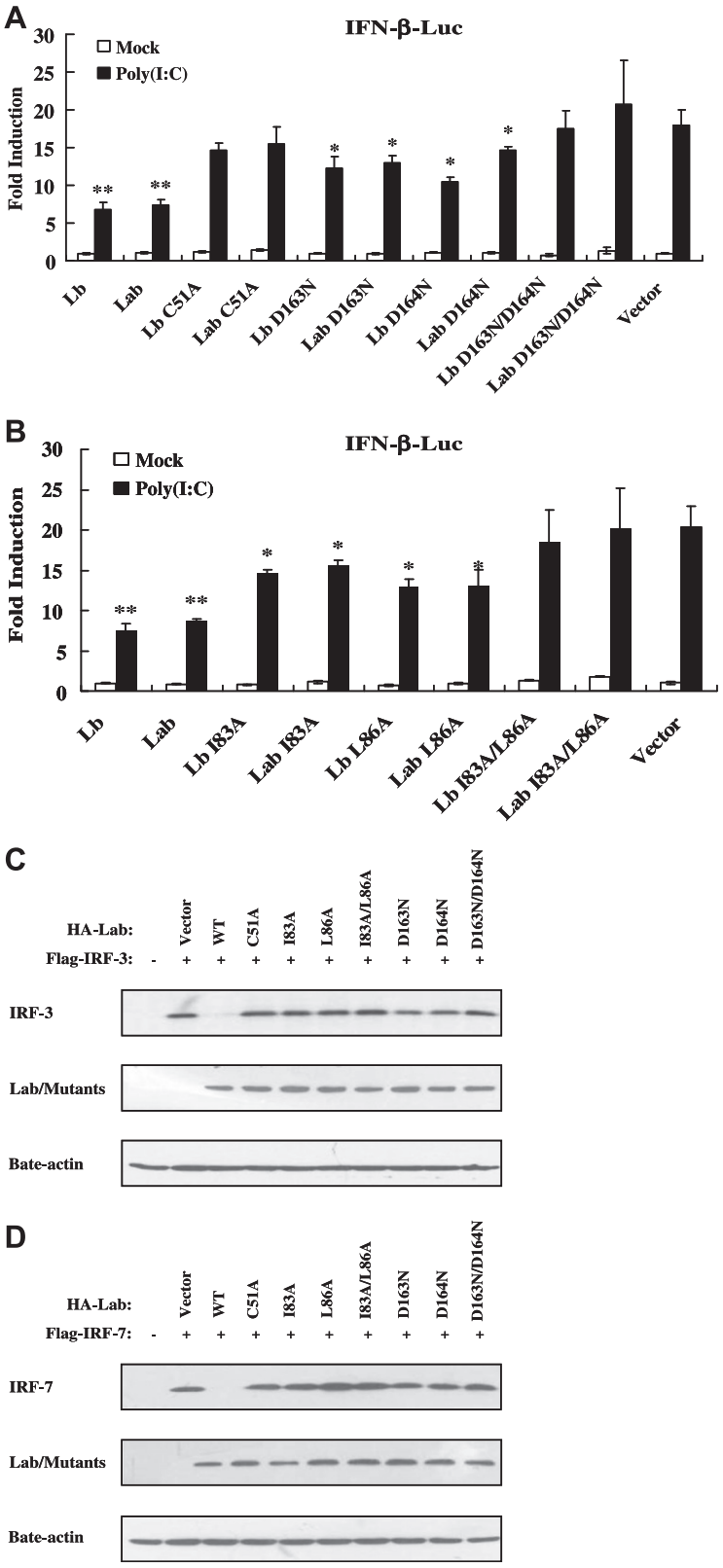

Fig. 4. The ability to process eIF-4G of L^{pro} was not necessary for suppressing dsRNA-induced activation of the IFN-α1/β promoter and decreasing IRF-3/7 in protein levels. (A,B) PK-15 cells were co-transfected with the IFN-β-Luc reporter plasmid (0.1 μg), 0.05 μg of pRL-TK, and the designated L^{pro} expression plasmids (1 μg). An empty vector (pCMV-HA) was used as a control. Twenty-four hours after the initial transfection, the cells were transfected with 1 μg of poly(I:C). Cell extracts were collected at 12 h after the second transfection and analyzed for firefly and Renilla luciferase expression. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with vector plus poly(I:C). (C,D) PK-15 cells were transfected with IRF-3 or IRF-7 (2 μg), along with indicated Lab^{pro} expression plasmids (1 μg). Cell lysates were prepared at 48 h post-transfection and analyzed for Flag-IRF-3/7 and HA-Lab^{pro} conjugated proteins by Western blot.

FMDV inhibits the activation of IRF-3/7, we further investigated whether the overexpression of L^{pro} inhibits MDA5-mediated activation of the IFN-α/β promoter. To this end, PK-15 cells were transfected with DNA constructs encoding porcine MDA5, IPS-1, IRF-3,

IRF-7, IRF-3(5D), or IRF-7(Δ243–451), together with IFN-β-Luc. As shown in Fig. 2A, overexpression of MDA5, IPS-1, IRF-3, IRF-7, IRF-3(5D), or IRF-7(Δ243–451) significantly activated the IFN-β promoter compared with the results using the empty plasmid transfection control. However, the activation of the IFN-β promoter by MDA5, IPS-1, IRF-3, IRF-7, IRF-3(5D), or IRF-7 (Δ243–451) was inhibited in the presence of L^{pro}. Similar results were obtained when the cells were transfected with IFN-α1-Luc or 4× PRDIII/I-Luc (data not shown) instead of IFN-β-Luc. Taken together, these results demonstrate that L^{pro} is sufficient to inhibit the IFN-α/β expression pathway induced by MDA5 at or downstream of IRF-3/7.

3.4. L^{pro} significantly reduced the transcription of multiple IRF-responsive genes

The IRFs transcription factor activates the expression of several genes, such as those for 2′,5′-OAS, ISG54, IP-10, and RANTES, whose promoters contain the IRFs binding domain [28,29]. To determine whether L^{pro} negatively influences the transcription of these genes, PK-15 cells were transfected with pHA-Lab or pHA-Lb. At 24 h after transfection, cells were further transfected with poly(I:C). RNAs were extracted from the cells and analyzed for 2′,5′-OAS, ISG54, IP-10, and RANTES mRNA expression by SYBR Green real-time RT-PCR. Poly(I:C) efficiently induced the expression of 2′,5′-OAS (Fig. 2B), ISG54 (Fig. 2C), IP-10 (Fig. 2D) and RANTES (Fig. 2E) in cells transfected with control vector. In contrast, expression of all the genes was inhibited in cells overexpressing either Lab^{pro} or Lb^{pro}. These results indicate that L^{pro} efficiently blocks the induction of genes whose transcription depends on IRFs.

3.5. L^{pro} decreases IRF-3/7 in protein levels

Since L^{pro} affect IRF-3/7-mediated activation of the IFN-α1/β promoter and IRF-responsive genes, we asked whether L^{pro} would decrease IRF-3/7 expression. No commercial porcine IRF-3/7 antibody are currently available, therefore we test whether L^{pro} induces the depletion of Flag-IRF-3/7 expressed from a CMV promoter. Interestingly, in Lab^{pro}-transfected cells, the constitutive expression of IRF-3/7 protein disappeared in a dose-dependent manner (Fig. 3A and B). Furthermore, overexpression of Lab^{pro} also decreases porcine p65 protein in a dose-dependent manner (Fig. 3C), this data was consistent with the report that L^{pro} inhibits IFN-β gene expression through degradation of p65/RelA, a subunit of NF-κB [10]. Similar results were observed in Lb^{pro}-expressing cells (data not shown). Our results indicate that both forms of L^{pro} are equally effective in decreasing IRF-3, IRF-7, and p65 in protein levels.

In view of the above-mentioned results, it was considered important to analyze whether L^{pro} would destabilize IRF-3/7 mRNA. To investigate this, we measured IRF-3/7 mRNA by real-time RT-PCR in RNA extracts from either mock or L^{pro}-transfected PK-15 cells. The results show that the IRF-3/7 mRNA content in mock-transfected cells was equal to the IRF-3/7 mRNA levels detected in L^{pro}-transfected cells (Fig. 3D and E). Taken together, these data demonstrated that L^{pro} regulates the IRF-3/7 protein turnover rather than mRNA transcription.

3.6. The ability to process eIF-4G of L^{pro} is not required to block poly(I:C)-mediated activation of the IFN-α1/β promoter

It is well known that L^{pro} possesses the ability to cleave the translation initiation factor eIF-4G and shuts off host cell translation [30]. We would like to know whether the L^{pro}-mediated IFN-α/β inhibition and IRF-3/7 depletion are depended on its ability to cleave eIF-4G. Previous studies have revealed that mutagenesis of amino acids C51, D163, and D164 in two forms of L^{pro} partially (D163N and D164N) or completely (C51A and D163N/D164N) abolished the ability of self-processing and eIF-4G cleavage compared with those of wild-type L^{pro} [30,31]. In addition, the disruption of a SAP domain with double amino acid substitutions at position 83 (I83) and 86 (L86) amino acids in L^{pro} did not affect L^{pro} enzymatic activity for self-processing and eIF-4G processing [32]. Based on these findings, we constructed mutants with single or multiple amino acid substitutions in the L^{pro}. Similar to the previous studies [30–32], with I83A/L86A, the ability of processing eIF-4G was still remain, while with C51A or D163N/D164N, there was no detectable eIF-4G cleavage remaining (data not shown). These mutants were then analyzed for their capacity to inhibit IFN-α1/β promoter activation and to decrease IRF-3/7 expression in PK-15 cells. As shown in Fig. 4A and B, overexpression of L^{pro} significantly suppressed IFN-β promoter activity induced by poly(I:C). However, both catalytic activity mutants (C51A and D163N/D164N) and double SAP domain mutants (I83A/L86A) abolished the capacity of L^{pro} to block signaling to the IFN-β promoter (Fig. 4A and B) and to decrease IRF-3/7 expression (Fig. 4C and D). Since double SAP domain mutants (I83A/L86A) possesses the ability to cleave eIF-4G, but failed to block IFN-β signaling pathway, suggesting that the ability to process eIF-4G of L^{pro} is not necessary to inhibit the IFN-β promoter induction and to decreases IRF-3/7 in protein levels. In addition, the catalytic activity mutants (C51A and D163N/D164N) did not inhibit the IFN-β promoter induction and decrease IRF-3/7 expression, demonstrating that the catalytic activity of L^{pro} is necessary for this function. Similarly, both the catalytic activity and SAP domain of L^{pro} were founded to be necessary for suppressing the host cell signaling to dsRNA-induced activation of IFN-α1 promoter (data not shown). Based on the previous studies [10,32] and our present results, it can be concluded that, in addition to inhibiting IFN-α/β protein synthesis, L^{pro} uses alternative mechanisms to decrease IRF-3/7 expression, as well as NF-κB, resulting in the interference in the induction of IFN-α1/β mRNA transcription. To identify the underlying mechanisms used by L^{pro} is underway in our laboratory.

4. Conclusions

Our data clearly demonstrate that FMDV L^{pro} alone, in the absence of other FMDV proteins, inhibits dsRNA-induced IFN-α/β expression. In addition to disrupting NF-κB, L^{pro} also inhibits the activation of IRF-3/7 through a decrease of IRF-3/7 in protein levels, which in turn, affects the full expression of IFN-α/β and other inflammatory cytokines, suggesting a global mechanism by which FMDV antagonizes the cellular innate immune and inflammatory responses to viral infection. In addition, our observations contribute to the understanding of the interaction between FMDV L^{pro} and the host by identifying regions within L^{pro} that may be responsible for the various functions of this protein. Understanding the relationship between L^{pro} and the host innate response should help develop more effective disease control strategies.

Acknowledgments

This work was supported by the National Basic Research Program (973) of China (2005CB523200), 863 High Technology R&D Project (2006AA10A204), and the New Century Excellent Talent Project (NCET-07-0347).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.044.

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