

The Leader Proteinase of Foot-and-Mouth Disease Virus Negatively Regulates the Type I Interferon Pathway by Acting as a Viral Deubiquitinase[∇]

Dang Wang,¹ Liurong Fang,¹ Ping Li,¹ Li Sun,² Jinxiu Fan,¹ Qingye Zhang,³ Rui Luo,¹ Xiangtao Liu,⁴ Kui Li,⁵ Huanchun Chen,¹ Zhongbin Chen,^{2*} and Shaobo Xiao^{1*}

Division of Animal Infectious Diseases, State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China¹; Division of Infection and Immunity, Department of Electromagnetic and Laser Biology, Beijing Institute of Radiation Medicine, Beijing 100850, China²; National Engineering Research Centre of Microbial Pesticides, Huazhong Agricultural University, Wuhan, 430070, China³; State Key Laboratory of Veterinary Etiology Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu 730046, China⁴; and Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, Tennessee 38163⁵

Received 14 December 2010/Accepted 25 January 2011

The leader proteinase (L^{pro}) of foot-and-mouth disease virus (FMDV) is a papain-like proteinase that plays an important role in FMDV pathogenesis. Previously, it has been shown that L^{pro} is involved in the inhibition of the type I interferon (IFN) response by FMDV. However, the underlying mechanisms remain unclear. Here we demonstrate that FMDV Lb^{pro}, a shorter form of L^{pro}, has deubiquitinating activity. Sequence alignment and structural bioinformatics analyses revealed that the catalytic residues (Cys51 and His148) are highly conserved in FMDV Lb^{pro} of all seven serotypes and that the topology of FMDV Lb^{pro} is remarkably similar to that of ubiquitin-specific protease 14 (USP14), a cellular deubiquitylation enzyme (DUB), and to that of severe acute respiratory syndrome coronavirus (SARS-CoV) papain-like protease (PLpro), a coronaviral DUB. Both purified Lb^{pro} protein and *in vivo* ectopically expressed Lb^{pro} removed ubiquitin (Ub) moieties from cellular substrates, acting on both lysine-48- and lysine-63-linked polyubiquitin chains. Furthermore, Lb^{pro} significantly inhibited ubiquitination of retinoic acid-inducible gene I (RIG-I), TANK-binding kinase 1 (TBK1), TNF receptor-associated factor 6 (TRAF6), and TRAF3, key signaling molecules in activation of type I IFN response. Mutations in Lb^{pro} that ablate the catalytic activity (C51A or D163N/D164N) or disrupt the SAP (for SAF-A/B, Acinus, and PIAS) domain (I83A/L86A) abrogated the DUB activity of Lb^{pro} as well as its ability to block signaling to the IFN- β promoter. Collectively, these results demonstrate that FMDV Lb^{pro} possesses DUB activity in addition to serving as a viral proteinase and describe a novel mechanism evolved by FMDV to counteract host innate antiviral responses.

Foot-and-mouth disease (FMD) is a highly contagious viral disease of wild and domestic cloven-hoofed animals (22). 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 encodes a polyprotein which is processed into structural and nonstructural proteins by three virus-encoded proteinases, i.e., leader (L^{pro}), 2A, and 3C^{pro} (31). L^{pro}, the first protein to be translated from the FMDV genome, is initiated at two different AUGs that are separated by 84 nucleotides, and this results in two alternative forms of L^{pro}, termed Lab^{pro} and Lb^{pro}. Both forms have been detected *in vitro* and in infected cells (7, 32). L^{pro} is a well-characterized papain-like protease that cleaves itself off the

nascent polyprotein precursor (36, 39). Using a genetically engineered FMDV lacking the L^{pro}-coding region (A12-LLV2), de Los Santos et al. demonstrated that L^{pro} inhibits the induction of beta interferon (IFN- β) and blocks the host innate immune response (9). However, the exact molecular mechanisms underlying the ability of L^{pro} to inhibit IFN- β induction remain to be elucidated.

It is well known that IFN- β transcription requires the coordinate activation of the latent transcription factors NF- κ B, interferon regulatory factors (IRFs), and ATF2-c-Jun (AP-1) and their subsequent binding to the IFN- β enhancer elements (47). Many of the signaling events that link the sensors to the transcription factors are mediated by the activities of kinases and ubiquitinating enzymes that modify and activate critical intermediates in the signaling cascade (3, 4, 25). Type I IFNs trigger signals that culminate in expression of IFN-stimulated genes (ISGs) and inflammatory cytokines, which suppress the replication of invading pathogens and also facilitate the development of adaptive immune responses (37, 48). However, the ubiquitin (Ub) chains conjugated to signaling molecules during activation of each pathway can be inactivated by cellular deubiquitylation enzymes (DUBs) such as A20, CYLD, and

* Corresponding author. Mailing address for Shaobo Xiao: Laboratory of Infectious Diseases, College of Veterinary Medicine, Huazhong Agricultural University, 1 Shi-zi-shan Street, Wuhan 430070, China. Phone: 86-27-8728 6884. Fax: 86-27-8728 2608. E-mail: vet@mail.hzau.edu.cn. Mailing address for Zhongbin Chen: Division of Infection and Immunity, Department of Electromagnetic and Laser Biology, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China. Phone: 86-10-6693 0297. Fax: 86-10-8827 2105. E-mail: chenzb@bmi.ac.cn.

[∇] Published ahead of print on 9 February 2011.

DUBA (17, 26, 52), suggesting that ubiquitin modification enzymes and DUBs play critical roles in modulating the immune responses.

Recent work has further revealed that many viruses have evolved elaborate strategies to counteract innate antiviral immune signaling pathways by redirecting or inhibiting the ubiquitination machinery of the host for their survival (49). For example, HIV-1 prevents the antiviral interferon response via Vpr- and Vif-directed ubiquitin-mediated degradation of IRF-3 (35), the N-terminal protease (Npro) of bovine viral diarrhoea virus interacts with IRF-3 and promotes its polyubiquitination and degradation through the proteasome (5, 23), and the murid herpesvirus 4 (MuHV-4) latency-associated protein ORF73 associates with the host ubiquitin-ligase complex to promote polyubiquitination and subsequent proteasomal degradation of p65/RelA, which inhibits the activity of NF- κ B that facilitates MuHV-4 latency (40). Recent studies showed that the papain-like protease (PLpro) domains of many coronaviruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), human coronavirus (HCoV) NL63, and mouse hepatitis virus (MHV) A59, act as both papain-like proteases and deubiquitinating enzymes that block type I IFN induction (2, 6, 8, 13, 18, 28, 54). The FMDV L^{PRO} is a papain-like protease and has been shown to inhibit the induction of transcription of IFN- β (9, 10, 39, 51). However, the precise mechanism(s) by which FMDV L^{PRO} exerts this effect remains unclear. In this work, sequence alignment and structural bioinformatics analyses suggested that the topology of FMDV Lb^{PRO} is remarkably similar to that of ubiquitin-specific protease 14 (USP14) (24), a cellular DUB, and to that of SARS-CoV PLpro (38), a coronaviral DUB. We then conducted experiments to demonstrate that the papain-like protease of FMDV, Lb^{PRO}, is also a novel viral DUB. Furthermore, we found that Lb^{PRO} cleaves ubiquitin moieties from critical signaling proteins of the type I IFN signaling pathway, such as retinoic acid-inducible gene I (RIG-I), TANK-binding kinase 1 (TBK1), tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3), and TRAF6. In addition, mutations that ablate the catalytic activity or disrupt the SAP (for SAF-A/B, Acinus, and PIAS) domain of Lb^{PRO} abrogate the DUB activity and also the ability of Lb^{PRO} to block IFN- β induction.

MATERIALS AND METHODS

Cells and virus. HEK293T cells (human embryonic kidney epithelial cells) were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% heated-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 10 μ g/ml streptomycin sulfate at 37°C in a humidified 5% CO₂ incubator. Porcine kidney (IBRS-2) cells were grown in Eagle minimal essential medium (MEM) supplemented with 10% heated-inactivated FCS, 100 U/ml penicillin, and 10 μ g/ml streptomycin sulfate. FMDV strain O/ES/2001 was propagated in IBRS-2 cells, and the supernatants of infected cells were clarified and stored at -80°C. Sendai virus (SEV) was obtained from the Centre of Virus Resource and Information, Wuhan Institute of Virology, Chinese Academy of Sciences.

Plasmids. Full-length hemagglutinin (HA)-tagged ubiquitin (Ub) plasmid (HA-Ub) and HA-Ub mutants in which all but one Lys residue (HA-K48-Ub or HA-K63-Ub) was replaced with Arg were gifts of Tomohiko Ohta (St. Marianna University School of Medicine, Japan) (34). pcDNA3.1-Flag-Ub was previously described (8). The expression plasmids for wild-type (WT) RIG-I (pEF-Flag-RIG-I), its constitutively active mutant (pEF-Flag-RIG-IN), and p125-Luc (IFN- β -Luc) were kindly provided by T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). The TBK1 expression vector was kindly provided by Himanshu Kuma and Shizuo Akira (Immunology Frontier Research

Center, Osaka University, Japan), The TRAF3 and TRAF6 expression vectors were gifts from Edward W. Harhaj (University of Miami School of Medicine, Miami, FL).

For construction of pcDNA3.1-V5/His/Lb^{PRO}, the cDNA fragment encoding the full-length Lb^{PRO} of FMDV was amplified by PCR from the cDNA of FMDV O/ES/2001 (GenBank accession no. AY686687) and subcloned into the pcDNA3.1-V5/His B vector (Invitrogen). Mutagenesis of individual amino acid residues (C51A, I83A, L86A, D163N, and D164N) in Lb^{PRO} was conducted using overlap extension PCR. Detailed sequences of the specific primers used are available upon request. All constructs were validated by DNA sequencing.

In vitro deubiquitination assay. The FMDV Lb^{PRO} protein was purified from cells transfected with pcDNA3.1-V5/His/Lb^{PRO} using Ni Sepharose 6 Fast Flow (GE Healthcare) according to the manufacturer's protocol. As a negative control, Ni Sepharose 6 Fast Flow was also used to isolate the proteins from empty-vector-transfected cells. The polyubiquitin chains were purchased from Boston Biochem (K48-Ub2-7 [catalog no. UC-230] and K63-Ub2-7 [catalog no. UC-330]). The purified products were incubated with 3 μ g of K48-Ub2-7 chains or K48-Ub2-7 chains at 37°C in a 30- μ l reaction mixture containing 25 mM NaCl, 100 mg/ml bovine serum albumin (BSA), and 2 mM dithiothreitol (DTT). A control reaction mixture was incubated under identical conditions with the exclusion of enzyme. Reactions were terminated by addition of 5 \times SDS-PAGE sample loading buffer (Beyotime, China) followed by heat treatment at 100°C for 5 min. The samples were analyzed by electrophoresis on a 15% SDS-polyacrylamide gel and stained with Coomassie blue dye.

Assay of deubiquitination activity in vivo. The effect of FMDV Lb^{PRO} on ubiquitinated cellular proteins *in vivo* was assessed as described previously (14). HEK293T cells cultured in 60-mm dishes were cotransfected with 1 μ g of HA-Ub, HA-K48-Ub, or HA-K63-Ub plus appropriate amounts of constructs containing FMDV Lb^{PRO} or the corresponding mutants using Lipofectamine 2000. Where applicable, the empty pcDNA3.1/V5-HisB vector was supplemented to keep the total amount of DNA transfected constant. After 30 h, cells were harvested by adding 250 μ l 2 \times lysis buffer A (LBA) (65 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 3% DL-dithiothreitol, and 40% glycerol) containing 20 mM *N*-ethylmaleimide (NEM) (Sigma) and 20 mM iodoacetamine (Sigma). Cell lysates were then analyzed for ubiquitin-conjugated proteins by Western blotting with anti-HA antibody (1:1,000) (MBL, Japan). To confirm the expression levels of FMDV Lb^{PRO} and the mutants, anti-V5 antibody (MBL, Japan) was used to detect the V5-tagged proteins. Beta-actin was detected with anti-beta-actin monoclonal antibody (MAb) (Beyotime, China) to demonstrate equal protein sample loading.

Luciferase reporter gene assay. HEK293T cells grown in 24-well plates were cotransfected with 0.1 μ g/well of IFN- β -Luc along with 0.05 μ g/well of pRL-TK plasmid (Promega) (for normalization of transfection efficiency) and various other expression plasmids or an empty control plasmid. In some experiments, cells were further infected or mock infected with SEV at 24 h after the initial cotransfection. Cells were harvested 12 h later, and firefly luciferase and *Renilla* luciferase activities were determined using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol. Data represent relative firefly luciferase activity normalized to *Renilla* luciferase activity and are representative of three independently conducted experiments. Data are presented as means \pm standard deviations (SD). A *P* value of less than 0.01 was considered highly statistically significant.

Coimmunoprecipitation and immunoblot analysis. Transient transfection of HEK293T cells with the indicated plasmids was performed routinely using Lipofectamine 2000 as per the manufacturer's instructions (Invitrogen). Transfected HEK293T cells from each 100-mm dish were lysed in 1 ml lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 20 mM phenylmethylsulfonyl fluoride [PMSF]), and the protein concentration was measured and adjusted. For each immunoprecipitation, 500 μ g of cell lysate protein was incubated with 0.5 μ g of the indicated antibody and 25 μ l of protein A+G-agarose (Beyotime, China) overnight at 4°C. The Sepharose beads were then washed three times with 1 ml lysis buffer. The precipitates were subjected to 10% SDS-PAGE and subsequent immunoblot analysis using the indicated antibodies.

RESULTS

Bioinformatics analysis predicts FMDV Lb^{PRO} to be a viral DUB. Based on the structures of their catalytic domains, the human DUBs have been classified into five subfamilies, most of which exhibit a high degree of homology mainly in two regions known as Cys and His boxes (C and H boxes, respec-

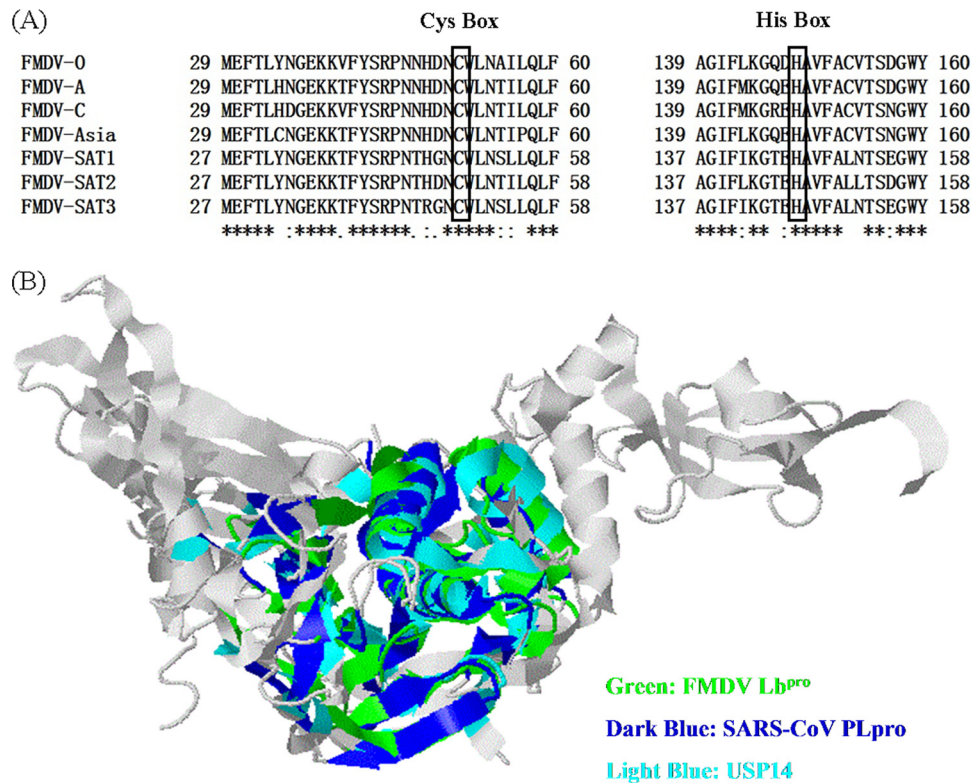


FIG. 1. Structure analysis of FMDV Lb^{Pro}. (A) Amino acid alignment of the conserved region surrounding Cys51 and His148 (numbering based on Lab^{Pro}) in the Lb^{Pro} proteins of the seven types of sequenced FMDV genomes. Black boxes indicate conserved enzymatic proteolysis residues. The sequences were derived from GenBank entries with the following accession numbers: FMDV type O, NC_004004; FMDV type A, NC_011450; FMDV type C, NC_002554; FMDV type Asia 1, NC_004915; FMDV type SAT1, NC_011451; FMDV type SAT2, NC_003992; and FMDV type SAT3, NC_011452. (B) Comparison of FMDV Lb^{Pro} (Protein Data Bank [PDB] code 1QOL) with SARS-CoV PLpro (PDB code 3E9S) and the cellular DUB USP14 (PDB code 2AYN). The topologies of the proteins were structurally aligned and superimposed using the Web-based server SSM (<http://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmsserver>). A ribbon diagram of conserved topologies shows FMDV Lb^{Pro}, SARS-CoV PLpro, and USP14 in color; the rest is represented by gray.

tively) that surround the catalytic Cys and His residues (33, 53). FMDV L^{Pro} is a well-characterized papain-like proteinase that also possesses the catalytic Cys and His residues (21, 43). Sequence alignment showed that Cys51 and His148 (numbering based on FMDV type O Lab^{Pro}) are highly conserved among all seven serotypes of FMDV (Fig. 1A). To predict whether FMDV Lb^{Pro} has the structural characteristics of a deubiquitinating enzyme, the structure comparison service Secondary-Structure Matching (SSM) (<http://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmsserver>) was used by clustering FMDV Lb^{Pro} with USP14 (24), a known cellular DUB, and SARS-CoV PLpro (38), a known viral DUB. As shown in Fig. 1B, the topology of FMDV Lb^{Pro} is remarkably similar to those of SARS-CoV PLpro and USP14, with corresponding root mean square (RMS) deviations of alignment of 2.845 Å and 2.776 Å, respectively, despite the fact that FMDV Lb^{Pro} has low amino acid sequence homology with SARS-CoV PLpro and USP14 (9.2% and 12.3%, respectively). These data suggested that FMDV Lb^{Pro} is likely a viral DUB.

FMDV Lb^{Pro} processes K48-linked and K63-linked polyubiquitin *in vitro*. As mentioned in the introduction, initiation of FMDV protein synthesis can occur at the first or the second AUG codon (7, 32). To avoid initiation of translation at both sites, we subcloned the FMDV Lb^{Pro} fragment into pcDNA3.1-

V5/His B in frame with C-terminal V5-6× His tags and transfected the construct into HEK293T cells. To further determine if FMDV Lb^{Pro} has DUB activity, a DNA construct expressing FMDV Lb^{Pro} was transiently transfected into HEK293T cells and the recombinant Lb^{Pro} was purified from cell lysates using Ni Sepharose 6 Fast Flow (Fig. 2A). When incubated with K48- and K63-linked polyubiquitin chains *in vitro*, the purified Lb^{Pro} was able to cleave both substrates (Fig. 2B and C). Upon prolonged incubation, Lb^{Pro} completely processed both substrates to monoubiquitin (data not shown). These data show that both of the two major forms of polyubiquitin chains, K48 and K63, serve as *in vitro* substrates for recombinant FMDV Lb^{Pro}.

FMDV Lb^{Pro} has DUB activity *in vivo*. To determine whether Lb^{Pro} functioned as a DUB in a cell-based assay, HEK293T cells were transfected with the empty vector or increasing amounts of plasmid DNA encoding Lb^{Pro} along with HA-tagged ubiquitin vector (HA-Ub), and the effect of Lb^{Pro} on all ubiquitinated cellular proteins was assessed via Western blotting with an anti-HA antibody. As shown in Fig. 3A, expression of Lb^{Pro} resulted in a dose-dependent reduction in the level of ubiquitinated cellular proteins compared to that in the control vector-transfected cells. To further identify which Ub linkage type is targeted by Lb^{Pro} *in vivo*, HEK293T cells were

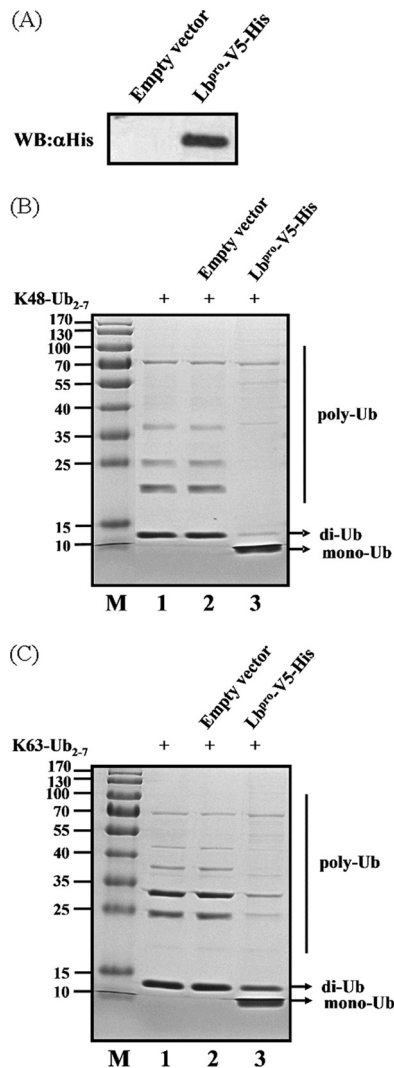


FIG. 2. Processing of K48- and K63-linked polyubiquitin chains by FMDV Lb^{PRO} *in vitro*. (A) The protein was obtained from FMDV Lb^{PRO}-transfected or mock-transfected 293T cells using Ni Sepharose 6 Fast Flow and analyzed for His-tagged FMDV Lb^{PRO}-conjugated proteins by Western blotting (WB) with an anti-His antibody. (B) *In vitro* K48-linked polyubiquitin deconjugation assay. K48-linked polyubiquitin was incubated with the protein obtained from mock-transfected (lane 2) or FMDV Lb^{PRO}-transfected (lane 3) HEK293T cells at 37°C for 1 h before being analyzed by SDS-PAGE. Lane 1, uncleaved K48-linked polyubiquitin chain (K48-Ub₂₋₇). M, molecular mass markers, including 170-, 130-, 100-, 70-, 55-, 40-, 35-, 25-, 15-, and 10-kDa bands. (C) *In vitro* K63-linked polyubiquitin deconjugation assay. The experiment was performed similarly to that described for panel B except that the K63-linked polyubiquitin chain (K63-Ub₂₋₇) was used.

transfected with HA-K48-Ub or HA-K63-Ub in lieu of HA-Ub. These constructs allow solely the formation of K48- and K63-linked polyubiquitin chains, respectively (34). Notably, both K48- and K63-linked Ub chains were processed by Lb^{PRO} in a dose-dependent manner, with no apparent preference between them (Fig. 3B and C). These results confirm our earlier results that FMDV Lb^{PRO} is a potent DUB that removes ubiquitin conjugates formed through either K48 or K63 linkage from many cellular substrates.

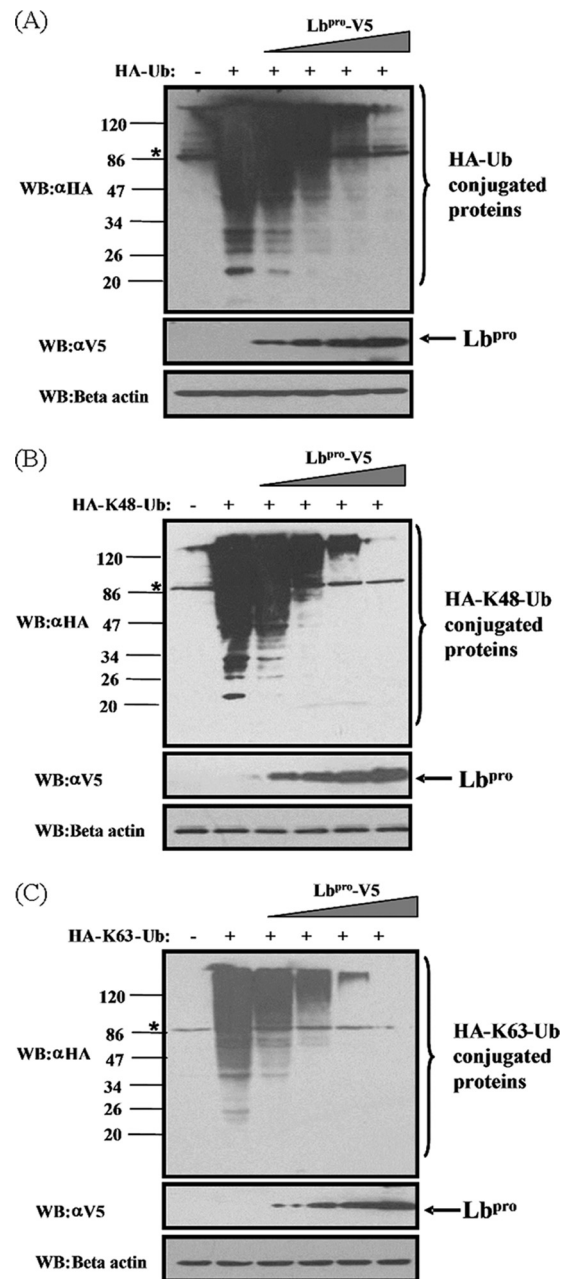


FIG. 3. FMDV Lb^{PRO} has a dose-dependent deubiquitinating activity *in vivo*. (A) HEK293T cells grown in 60-mm dishes were transfected with HA-tagged Ub expression plasmids (1.2 μg), along with increasing quantities (0, 0.008, 0.04, 0.2, or 1 μg) of plasmid encoding Lb^{PRO}, using Lipofectamine 2000. Cell lysates were prepared at 30 h post-transfection and analyzed for Ub-conjugated proteins by Western blotting with an anti-HA antibody. Western blotting with anti-V5 antibody shows expression of Lb^{PRO}, and Western blotting for beta-actin serves as a protein loading control. (B and C) FMDV Lb^{PRO} effectively cleaves both K48 and K63 Ub linkages *in vivo*. The experiment was performed similarly to that described for panel A except that HA-K48-Ub or HA-K63-Ub was used in lieu of HA-Ub. The asterisk indicates a nonspecific band.

To exclude the possibility that the DUB activity of Lb^{PRO} observed was an artificial effect of plasmid overexpression in cell culture, we analyzed the DUB activity in the context of FMDV infection. IBRS-2 cells were transfected with the

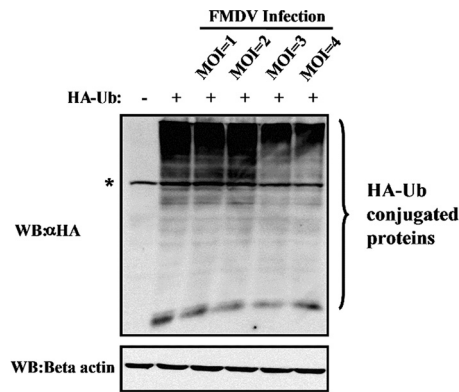


FIG. 4. DUB activity assay during FMDV infection. IBRS-2 cells cultured in 60-mm dishes were transfected with HA-tagged ubiquitin and infected with FMDV at different MOIs at 24 h posttransfection. Cells were lysed at 12 h postinfection and assayed for anti-HA and anti-beta-actin staining by Western blotting. The asterisk indicates a nonspecific band.

HA-Ub vector for 24 h and then infected with FMDV at increasing multiplicities of infection (MOIs). As shown in Fig. 4, compared to those in uninfected cells, the levels of ubiquitinated cellular proteins were reduced in a dose-dependent manner in FMDV-infected cells. Similar results were obtained when K48-Ub or K63-Ub was transfected in place of HA-Ub prior to FMDV infection (data not shown). In aggregate, these data suggest that Lb^{pro}, expressed from replicating FMDV, possesses DUB activities toward both K48- and K63-linked ubiquitin chains.

The DUB activity of FMDV Lb^{pro} is uncoupled from its ability to process eIF-4G. It is established that L^{pro} possesses the ability to cleave the translation initiation factor eIF-4G and shuts off host cell translation (15). We considered the possibility that the DUB activity of Lb^{pro} is coupled with or dependent upon its ability to cleave eIF-4G. Previous studies revealed that mutations in amino acid residues C51, D163, and D164 in L^{pro} partially reduced (D163N and D164N) or completely eliminated (C51A and D163N/D164N) the abilities of L^{pro} to process itself from viral polyprotein and to cleave eIF-4G (15, 41). Such activities of L^{pro}, however, were not affected upon disruption of the SAP domain by double amino acid substitutions at residues 83 and 86 (I83A/L86A) (11). Based on these previous findings, we constructed these Lb^{pro} mutants and compared them with WT Lb^{pro} for DUB activity and the ability to cleave eIF-4G. In agreement with the previous studies, the I83A/L86A double mutant retained the ability to cleave eIF-4G (Fig. 5A, lane 7), while the D163N/D164N double mutant (lane 10) and the C51A mutant (lane 4) completely lost such activity. These Lb^{pro} mutants were then examined for DUB activity in HEK293T cells. To this end, HEK293T cells were cotransfected with the Flag-Ub vector and the indicated Lb^{pro} expression plasmids. As shown in Fig. 5B, overexpression of WT Lb^{pro} almost completely eliminated Flag-Ub-reactive protein bands (lane 3). The catalytically inactive mutants (C51A and D163N/D164N) lost the DUB activity (lanes 4 and 10, respectively), as did the SAP domain I83A/L86A double mutant (lane 7), which is capable of eIF-4G cleavage. These data suggest that although the catalytic activity

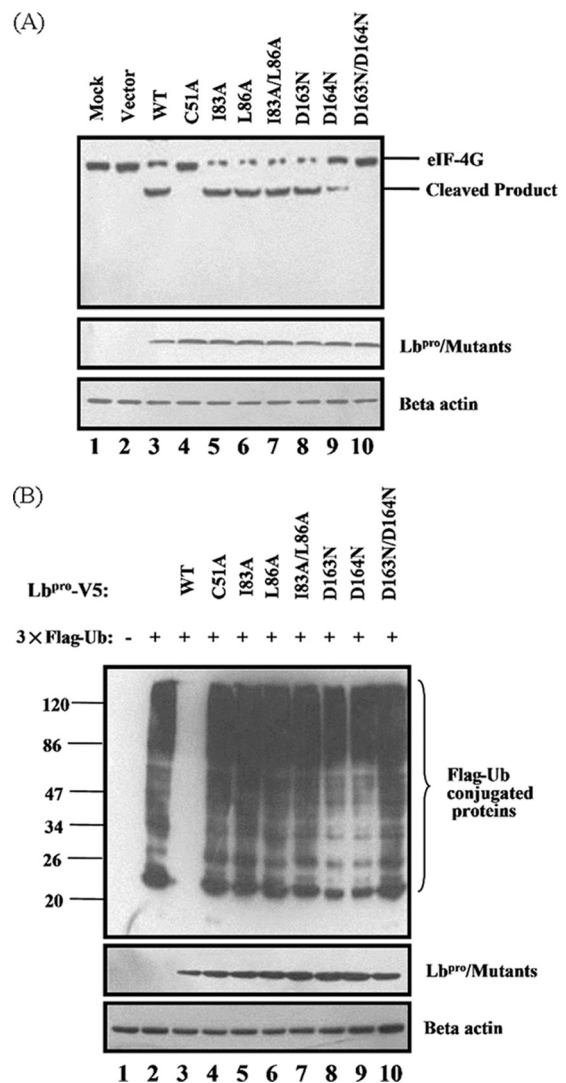


FIG. 5. The ability of Lb^{pro} to process eIF-4G is not necessary for its DUB activity. (A) HEK293T cells cultured in 60-mm dishes were transfected with the indicated Lb^{pro} expression plasmids (2 μg). Cell lysates were prepared at 30 h posttransfection and analyzed for cellular eIF-4G proteins with anti-eIF-4G antibody (Cellsignal, catalog no. 2498) by Western blotting. (B) HEK293T cells cultured in 60-mm dishes were transfected with Flag-tagged Ub expression plasmid (pcDNA3.1-Flag-Ub) (1.2 μg), along with indicated Lb^{pro} expression plasmids (1 μg). Cell lysates were prepared at 30 h posttransfection and analyzed for Flag-Ub and Lb^{pro}-V5 conjugated proteins by Western blotting. Anti-beta-actin antibody was used to detect beta-actin, which serves as a protein loading control.

of Lb^{pro} is important for its DUB activity, the latter does not depend on the ability of Lb^{pro} to process eIF-4G (i.e., proteolytic activity). Further confirming this notion, the D164N mutant was less active in cleaving eIF-4G than was the I83A/L86A double mutant (Fig. 5A, compare lanes 9 and 7) but had substantially better DUB activity than the latter (Fig. 5B, compare lanes 9 and 7). Similar results were obtained when K48-Ub or K63-Ub was used in lieu of HA-Ub to compare the DUB activities of WT and mutant Lb^{pro} (data not shown).

DUB activity is essential for FMDV Lb^{pro} to block type I IFN induction. Ubiquitination is essential for the activation of

many components of the type I IFN signaling pathway, such as RIG-I, TBK1, TRAF3, and TRAF6 (12, 19, 20, 30, 50). In addition, the negative feedback regulation of these molecules depends in part on DUBs (25, 44, 49). Previous studies have shown that several viral proteins that contain a conserved DUB motif can block the IFN- β response, including SARS-CoV PLpro, HCoV NL63 PLP2, and MHV A59 PLP2 (8, 13, 18, 54). To determine whether Lb^{pro} also can block the type I IFN signaling pathway, HEK293T cells were transfected with an Lb^{pro} expression construct together with a luciferase reporter plasmid with the IFN- β promoter and pRL-TK, followed by SEV infection. As shown in Fig. 6A, Lb^{pro} downregulated SEV-induced IFN- β promoter in a dose-dependent manner; these data were consistent with the report that L^{pro} inhibits IFN- β transcription (9, 10, 51). We further investigated whether overexpression of Lb^{pro} inhibits RIG-I-, TBK1-, and TRAF6-mediated activation of the IFN- β promoter. To this end, HEK293T cells were transfected with DNA constructs encoding RIG-I, TBK1, or TRAF6, together with IFN- β -Luc. As shown in Fig. 6B, overexpression of RIG-I, TBK1, or TRAF6 significantly activated the IFN- β promoter compared with cells transfected with the empty vector control. However, such effects were all substantially reduced in the presence of Lb^{pro}.

To determine whether the DUB activity of Lb^{pro} is involved in Lb^{pro} inhibition of type I IFN induction, various Lb^{pro} mutants with differing DUB activities (Fig. 5B) were analyzed for their ability to impair signaling to the SEV-induced IFN- β promoter in HEK293T cells. As shown in Fig. 6C, the catalytically inactive mutants (C51A and D163N/D164N) devoid of DUB activity completely lost the ability to block viral activation of the IFN- β promoter, as did the proteolytically active, SAP domain I83A/L86A double mutant, which was also defective for DUB activity. Thus, the DUB activity, but not the proteolytic activities toward viral polyprotein and eIF-4G, is critical for the function of Lb^{pro} as an IFN antagonist. Our data also revealed that the D163N and D164N mutants were still able to significantly inhibit IFN induction (to similar extents), although both were less effective than WT Lb^{pro} (Fig. 6C). This provides further support for the notion that the ability of FMDV Lb^{pro} to block viral induction of IFN- β transcription is proportional to its DUB activity instead of the proteolytic activity, because these two mutants differed in their proteolytic activity (Fig. 5A, lanes 8 and 9) yet had similar reduced DUB activity compared with WT Lb^{pro} (Fig. 5B, lanes 8 and 9). We found that these Lb^{pro} mutants acted in a similar fashion when the IFN- β promoter was activated by ectopic expression of RIG-I, TBK1, and TRAF6 (data not shown).

FMDV Lb^{pro} deubiquitinates RIG-I, TBK1, TRAF3, and TRAF6. We further investigated whether the IFN antagonist function of Lb^{pro} is associated with the deubiquitination of RIG-I, TBK1, TRAF6, and TRAF3, which are essential signaling components in the type I IFN pathway activated by virus infection. We found that overexpression of Lb^{pro} significantly inhibited ubiquitination of RIG-I (Fig. 7A), TBK1 (Fig. 7B), TRAF6 (Fig. 7C), and TRAF3 (Fig. 7D). In contrast, the Lb^{pro} mutants (C51A, D163N/D164N, and I83A/L86A) lacking DUB activity (Fig. 5B) and incapable of inhibiting IFN induction (Fig. 6C) had no such effects. Taken together, our results

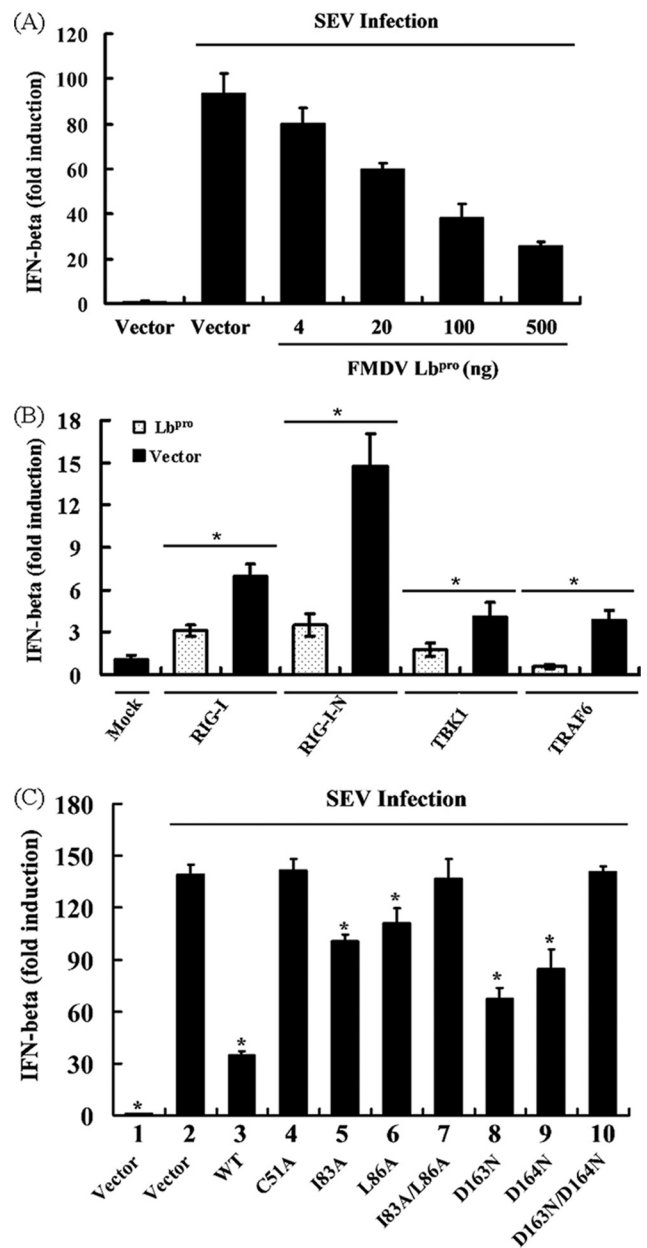


FIG. 6. Lb^{pro} inhibits type I interferon induction. (A) HEK293T cells grown in 24-well plates were transfected with 0.1 μ g/well of IFN- β -Luc reporter plasmid, along with 0.05 μ g/well of pRL-TK plasmid and increasing quantities (0, 0.004, 0.02, 0.1, or 0.5 μ g) of plasmid encoding Lb^{pro}, using Lipofectamine 2000. Twenty-four hours after the initial transfection, the cells were further infected with SEV or mock infected. Luciferase assays were performed at 18 h after infection. Results represent the means and standard deviations from 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 1. (B) HEK293T cells were cotransfected with the IFN- β -Luc reporter plasmid (0.1 μ g), pRL-TK plasmid (0.05 μ g), and 0.5 μ g of plasmid encoding Lb^{pro} together with the RIG-I, RIG-I-N, TBK1, or TRAF6 expression vector (0.5 μ g). Luciferase assays were performed at 36 h after transfection. (C) HEK293T cells were cotransfected with the IFN- β -Luc reporter plasmid (0.1 μ g), 0.05 μ g of pRL-TK, and the designated Lb^{pro} expression plasmids (0.5 μ g). An empty vector (pcDNA3.1-V5/His B) was used as a control. Twenty-four hours after the initial transfection, the cells were further infected with SEV or mock infected. Cell extracts were collected at 18 h after infection and analyzed for firefly and *Renilla* luciferase expression. *, $P < 0.01$ compared with vector plus SEV.

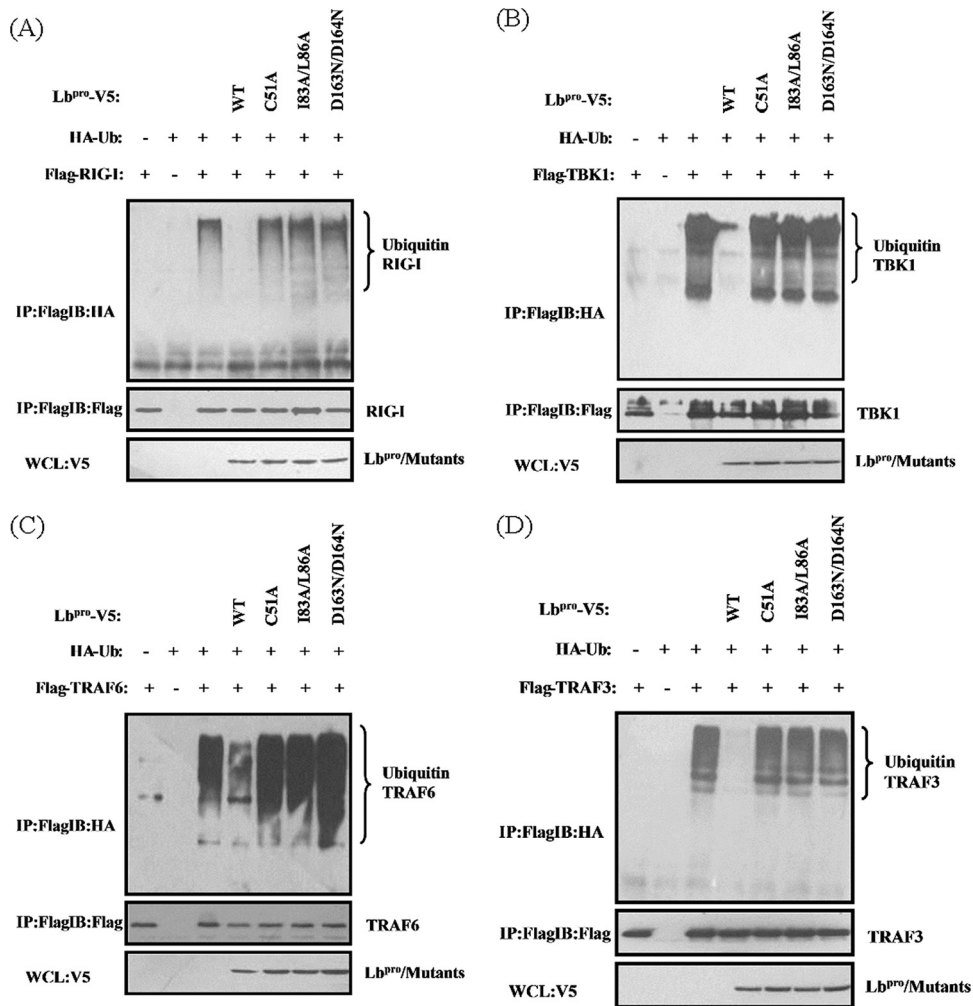


FIG. 7. FMDV Lb^{pro} inhibits ubiquitination of RIG-I, TBK1, TRAF6, and TRAF3. HEK293T cells cultured in 100-mm dishes were cotransfected with HA-tagged Ub expression plasmid (1.5 μg), 0.5 μg of the indicated plasmids encoding Lb^{pro}, and the RIG-I (A), TBK1 (B), TRAF6 (C), or TRAF3 (D) expression vector (4 μg). MG132 (20 nM) was added at 30 h after transfection. Cell lysates were prepared at 4 h after treatment and immunoprecipitated with anti-Flag antibody (Mabgene, China), and ubiquitin conjugation of protein was verified by immunoblotting with anti-HA antibody. The input tagged proteins were verified with the indicated antibodies.

suggest that the DUB activity of FMDV Lb^{pro} was pivotal for the inhibition of type I IFN induction.

DISCUSSION

FMD is one of the most contagious diseases of cloven-hoofed animals. The responsible agent, FMDV, rapidly replicates and disseminates within the infected animal and is able to spread quickly to susceptible animals that are in close contact (22). Previous studies have revealed that FMDV L^{pro}, a papain-like proteinase, is involved in antagonizing the innate immune responses by inhibiting type I interferon production, which is thought to play an important role in FMDV pathogenesis and virulence (10, 39, 51). Precisely how L^{pro} accomplishes this important function, however, remains unclear. Various mechanisms have been proposed, including the blockade of cap-dependent translation of cellular mRNAs (including those of IFNs) through L^{pro}-mediated cleavage of eIF-4G and the inhibition of induction of type I IFN transcription via an as-yet

unclear mechanism(s) (9, 10, 15, 51). In this study, we provide biochemical and molecular evidence that FMDV Lb^{pro} is a novel viral deubiquitinating enzyme. Our study also uncovers a novel mechanism by which FMDV Lb^{pro} antagonizes type I IFN induction, i.e., by deubiquitinating the critical signaling components RIG-I, TBK1, TRAF6, and TRAF3. Our data comparing various Lb^{pro} mutants suggest that the DUB activity of Lb^{pro}, but not its classical proteolytic activity toward eIF-4G and viral polyprotein, governs the ability of Lb^{pro} to block induction of the IFN-β promoter.

DUB activity was recently demonstrated for many viral proteins encoded by distinct viruses, such as human adenovirus, herpesvirus, coronavirus, and bunyavirus, and these enzymes play specific roles in regulation of viral infection (1, 2, 8, 16, 28, 42, 45, 54). Here, three strategies were used to demonstrate FMDV Lb^{pro} to be a novel viral DUB: (i) a bioinformatics approach to predict FMDV Lb^{pro} to be a DUB, (ii) an *in vitro* deubiquitination assay, and (iii) an assay of deubiquitination

activity *in vivo*. First, we found that FMDV Lb^{PRO} proteins of seven serotypes share highly conserved Cys and His residues and flanking regions, which exist in many human DUBs. Indeed, structure bioinformatic studies indicated that the topology of FMDV Lb^{PRO} is highly similar to those of USP14 and SARS-CoV PLpro, suggesting that FMDV Lb^{PRO} may have deubiquitinating activity. In subsequent experiments, we showed that Lb^{PRO} indeed had DUB activity both *in vitro* and *in vivo*. Importantly, we demonstrated that Lb^{PRO} expressed from viral polyprotein in the context of FMDV infection possessed DUB activity, confirming the biological relevance of this finding. Furthermore, we revealed that FMDV Lb^{PRO} could act on both K48- and K63-linked Ub polymers, a feature shared by other known viral DUBs, such as human cytomegalovirus (HCMV) UL48 (27), herpes simplex virus type 1 (HSV-1) UL36 (27), and SARS-CoV PLpro (29).

Many cysteine proteases encoded by RNA viruses, which generate mature viral proteins from viral polyprotein that are necessary for virus replication, have been found to be multifunctional proteins. Like the coronaviral papain-like proteases, including SARS-CoV PLpro, MHV A59 PLP2, and HCoV NL63 PLP2, and the cysteine protease of an arterivirus, i.e., the nonstructural protein 2 (NSP2) of porcine reproductive and respiratory syndrome virus, Lb^{PRO} possesses deubiquitination activity and is able to antagonize innate immune induction of type I interferon (2, 6, 8, 13, 18, 28, 45, 54). Using an IFN- β promoter reporter assay, we also demonstrated that Lb^{PRO}, as a novel picornavirus DUB, significantly blocked SEV-induced IFN- β expression in a dose-dependent manner. These data are consistent with previous reports that L^{PRO} is an antagonist of IFN- β (9, 10, 51). Recent studies indicated that a SAP domain exists in L^{PRO} (11). Initially, we would like to know which one of the three functions/domains of L^{PRO}, namely, cysteine protease, the SAP domain, and DUB activity, is sufficient for the inhibition of viral induction of IFN- β transcription. Although we cannot exclude the possibility that the protease activity is also involved, our data clearly demonstrated that the ability to block activation of the IFN- β promoter correlated with the DUB activity of Lb^{PRO} but not its proteolytic activity (toward eIF-4G). At present, we do not know whether the SAP domain also contributes to inhibition of IFN induction by Lb^{PRO}, as the SAP domain mutants also were impaired/defective for DUB activity. Future investigation to identify SAP mutants that disrupt this domain but do not affect the DUB activity will be required.

Ubiquitination and deubiquitination are critically involved in virus-induced type I IFN signaling pathways. Several ubiquitin ligase enzymes have been found to regulate these processes (3, 25, 44). For example, ubiquitination of RIG-I by the E3 ubiquitin ligase TRIM25 is necessary and sufficient to activate VISA/IPS-1/MAVS/Cardif, which triggers the downstream signaling cascade to produce type I IFN (19). Nrdp1, as an E3 ligase for TBK1, interacts with TBK1 and promotes ubiquitination of TBK1, leading to TBK1 and IRF-3 activation (50). In addition, virus-triggered ubiquitination of TRAF3/6 by cIAP1/2 is essential for induction of IFN- β and the cellular antiviral response (30). However, a few cellular DUBs are known to negatively regulate type I IFN signaling pathways. A20, DUBA, and CYLD were found to target RIG-I, TRAF3, and TBK1, respectively, for deubiquitination, thereby func-

tioning as negative regulators of innate immune responses (17, 26, 52). In addition, the bacterial virulence factor YopJ is a deubiquitinating protease that acts on TRAF proteins to prevent or remove the ubiquitin conjugates required for signal transduction (46). To our knowledge, thus far there has been no direct demonstration of whether viral DUBs can remove ubiquitin chains from RIG-I, TBK1, and TRAF3/6, which in turn negatively regulates type I IFN induction. We report here that FMDV Lb^{PRO} significantly inhibits ubiquitination of RIG-I, TBK1, and TRAF3/6, which is essential for activation of type I IFN signaling. Both catalytically inactive mutants and double SAP domain mutants that are defective for DUB activity lost the capability of reducing the ubiquitinated RIG-I, TBK1, and TRAF3/6, indicating that the DUB activity of FMDV Lb^{PRO} is directly involved in the inhibition of type I IFN induction. Thus, FMDV L^{PRO} is a multifunctional protein that blocks the IFN antiviral response through multiple distinct mechanisms: (i) L^{PRO} cleaves the translation initiation factor eIF-4G and shuts off host cell translation, resulting in lower levels of IFN protein expression (15); (ii) L^{PRO} represses the transcription of IFN- β not only by inhibiting activation of NF- κ B but also by decreasing IRF-3/7 protein expression (10, 51); and (iii) L^{PRO} acts as a DUB that cleaves ubiquitin chains from RIG-I, TBK1, TRAF6, and TRAF3, thereby inhibiting the activation of type I IFN signaling (this study). The identification of FMDV L^{PRO} as a viral DUB reveals the multilayered counteracting of host defense by a picornaviral leader protein and opens new research avenues to develop effective new strategies that target L^{PRO} for control of FMDV infections.

ACKNOWLEDGMENTS

We thank H. Kuma, S. Akira, E. W. Harhaj, T. Fujita, A. Marchese, and T. Ohta for providing reporter plasmids and expression constructs.

This work was supported by the New Century Excellent Talent Project (NCET-07-0347), the Program for Changjiang Scholars and Innovative Research Team in the University of China (IRT0726), the National Natural Science Foundation of China (30870536 and 30972761), the National S&T Major Project (2008ZX10004-015), and the Beijing Municipal Natural Science Foundation (7092075).

REFERENCES

- Balakov, M. Y., M. Jaquinod, A. L. Haas, and J. Chroboczek. 2002. Deubiquitinating function of adenovirus proteinase. *J. Virol.* **76**:6323–6331.
- Barretto, N., et al. 2005. The papain-like protease of severe acute respiratory syndrome coronavirus has deubiquitinating activity. *J. Virol.* **79**:15189–15198.
- Bhoj, V. G., and Z. J. Chen. 2009. Ubiquitylation in innate and adaptive immunity. *Nature* **458**:430–437.
- Bibeau-Poirier, A., and M. J. Servant. 2008. Roles of ubiquitination in pattern-recognition receptors and type I interferon receptor signaling. *Cytokine* **43**:359–367.
- Chen, Z., et al. 2007. Ubiquitination and proteasomal degradation of interferon regulatory factor-3 induced by Npro from a cytopathic bovine viral diarrhea virus. *Virology* **366**:277–292.
- Chen, Z., et al. 2007. Proteolytic processing and deubiquitinating activity of papain-like proteases of human coronavirus NL63. *J. Virol.* **81**:6007–6018.
- Clarke, B. E., et al. 1985. Two initiation sites for foot-and-mouth disease virus polyprotein *in vivo*. *J. Gen. Virol.* **66**:2615–2626.
- Clementz, M. A., et al. 2010. Deubiquitinating and interferon antagonism activities of coronavirus papain-like proteases. *J. Virol.* **84**:4619–4629.
- de Los Santos, T., S. de Avila Botton, R. Weiblen, and M. J. Grubman. 2006. The leader proteinase of foot-and-mouth disease virus inhibits the induction of beta interferon mRNA and blocks the host innate immune response. *J. Virol.* **80**:1906–1914.
- de Los Santos, T., F. Diaz-San Segundo, and M. J. Grubman. 2007. Degradation of nuclear factor kappa B during foot-and-mouth disease virus infection. *J. Virol.* **81**:12803–12815.
- de los Santos, T., et al. 2009. A conserved domain in the leader proteinase

- of foot-and-mouth disease virus is required for proper subcellular localization and function. *J. Virol.* **83**:1800–1810.
12. **Deng, L., et al.** 2000. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**:351–361.
 13. **Devaraj, S. G., et al.** 2007. Regulation of IRF-3-dependent innate immunity by the papain-like protease domain of the severe acute respiratory syndrome coronavirus. *J. Biol. Chem.* **282**:32208–32221.
 14. **Evans, P. C., et al.** 2004. Zinc-finger protein A20, a regulator of inflammation and cell survival, has de-ubiquitinating activity. *Biochem. J.* **378**:727–734.
 15. **Foeger, N., W. Glaser, and T. Skern.** 2002. Recognition of eukaryotic initiation factor 4G isoforms by picornaviral proteinases. *J. Biol. Chem.* **277**:44300–44309.
 16. **Frias-Staheli, N., et al.** 2007. Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. *Cell Host Microbe* **2**:404–416.
 17. **Friedman, C. S., et al.** 2008. The tumour suppressor CYLD is a negative regulator of RIG-I-mediated antiviral response. *EMBO Rep.* **9**:930–936.
 18. **Frieman, M., K. Ratia, R. E. Johnston, A. D. Mesecar, and R. S. Baric.** 2009. Severe acute respiratory syndrome coronavirus papain-like protease ubiquitin-like domain and catalytic domain regulate antagonism of IRF3 and NF-kappaB signaling. *J. Virol.* **83**:6689–6705.
 19. **Gack, M. U., et al.** 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* **446**:916–920.
 20. **Gatot, J. S., et al.** 2007. Lipopolysaccharide-mediated interferon regulatory factor activation involves TBK1-IKKepsilon-dependent Lys(63)-linked polyubiquitination and phosphorylation of TANK/I-TRAF. *J. Biol. Chem.* **282**:31131–31146.
 21. **Gorbalenya, A. E., E. V. Koonin, and M. M. Lai.** 1991. Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha- and coronaviruses. *FEBS Lett.* **288**:201–205.
 22. **Grubman, M. J., and B. Baxt.** 2004. Foot-and-mouth disease. *Clin. Microbiol. Rev.* **17**:465–493.
 23. **Hilton, L., et al.** 2006. The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. *J. Virol.* **80**:11723–11732.
 24. **Hu, M., et al.** 2005. Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. *EMBO J.* **24**:3747–3756.
 25. **Isaacson, M. K., and H. L. Ploegh.** 2009. Ubiquitination, ubiquitin-like modifiers, and deubiquitination in viral infection. *Cell Host Microbe* **5**:559–570.
 26. **Kayagaki, N., et al.** 2007. DUBA: a deubiquitinase that regulates type I interferon production. *Science* **318**:1628–1632.
 27. **Kim, E. T., S. E. Oh, Y. O. Lee, W. Gibson, and J. H. Ahn.** 2009. Cleavage specificity of the UL48 deubiquitinating protease activity of human cytomegalovirus and the growth of an active-site mutant virus in cultured cells. *J. Virol.* **83**:12046–12056.
 28. **Lindner, H. A., et al.** 2005. The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme. *J. Virol.* **79**:15199–15208.
 29. **Lindner, H. A., et al.** 2007. Selectivity in ISG15 and ubiquitin recognition by the SARS coronavirus papain-like protease. *Arch. Biochem. Biophys.* **466**:8–14.
 30. **Mao, A. P., et al.** 2010. Virus-triggered ubiquitination of TRAF3/6 by cIAP1/2 is essential for induction of interferon-beta (IFN-beta) and cellular antiviral response. *J. Biol. Chem.* **285**:9470–9476.
 31. **Mason, P. W., M. J. Grubman, and B. Baxt.** 2003. Molecular basis of pathogenesis of FMDV. *Virus Res.* **91**:9–32.
 32. **Medina, M., E. Domingo, J. K. Brangwyn, and G. J. Belsham.** 1993. The two species of the foot-and-mouth disease virus leader protein, expressed individually, exhibit the same activities. *Virology* **194**:355–359.
 33. **Nijman, S. M., et al.** 2005. A genomic and functional inventory of deubiquitinating enzymes. *Cell* **123**:773–786.
 34. **Nishikawa, H., et al.** 2004. Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. *J. Biol. Chem.* **279**:3916–3924.
 35. **Okumura, A., et al.** 2008. HIV-1 accessory proteins VPR and Vif modulate antiviral response by targeting IRF-3 for degradation. *Virology* **373**:85–97.
 36. **Piccone, M. E., E. Rieder, P. W. Mason, and M. J. Grubman.** 1995. The foot-and-mouth disease virus leader proteinase gene is not required for viral replication. *J. Virol.* **69**:5376–5382.
 37. **Randall, R. E., and S. Goodbourn.** 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J. Gen. Virol.* **89**:1–47.
 38. **Ratia, K., et al.** 2006. Severe acute respiratory syndrome coronavirus papain-like protease: structure of a viral deubiquitinating enzyme. *Proc. Natl. Acad. Sci. U. S. A.* **103**:5717–5722.
 39. **Roberts, P. J., and G. J. Belsham.** 1995. Identification of critical amino acids within the foot-and-mouth disease virus leader protein, a cysteine protease. *Virology* **213**:140–146.
 40. **Rodrigues, L., et al.** 2009. Termination of NF-kappaB activity through a gammaherpesvirus protein that assembles an ECSS ubiquitin-ligase. *EMBO J.* **28**:1283–1295.
 41. **Schlick, P., J. Kronovet, B. Hampoelz, and T. Skern.** 2002. Modulation of the electrostatic charge at the active site of foot-and-mouth-disease-virus leader proteinase, an unusual papain-like enzyme. *Biochem. J.* **363**:493–501.
 42. **Schlieker, C., G. A. Korbel, L. M. Kattenhorn, and H. L. Ploegh.** 2005. A deubiquitinating activity is conserved in the large tegument protein of the herpesviridae. *J. Virol.* **79**:15582–15585.
 43. **Skern, T., I. Fita, and A. Guarne.** 1998. A structural model of picornavirus leader proteinases based on papain and bleomycin hydrolase. *J. Gen. Virol.* **79**:301–307.
 44. **Sun, S. C.** 2008. Deubiquitylation and regulation of the immune response. *Nat. Rev. Immunol.* **8**:501–511.
 45. **Sun, Z., Z. Chen, S. R. Lawson, and Y. Fang.** 2010. The cysteine protease domain of porcine reproductive and respiratory syndrome virus nonstructural protein 2 possesses deubiquitinating and interferon antagonism functions. *J. Virol.* **84**:7832–7846.
 46. **Sweet, C. R., J. Conlon, D. T. Golenbock, J. Goguen, and N. Silverman.** 2007. YopJ targets TRAF proteins to inhibit TLR-mediated NF-kappaB, MAPK and IRF3 signal transduction. *Cell. Microbiol.* **9**:2700–2715.
 47. **Thanos, D., and T. Maniatis.** 1995. Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. *Cell* **83**:1091–1100.
 48. **Theofilopoulos, A. N., R. Baccala, B. Beutler, and D. H. Kono.** 2005. Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu. Rev. Immunol.* **23**:307–336.
 49. **Viswanathan, K., K. Fruh, and V. DeFilippis.** 2010. Viral hijacking of the host ubiquitin system to evade interferon responses. *Curr. Opin. Microbiol.* **13**:517–523.
 50. **Wang, C., et al.** 2009. The E3 ubiquitin ligase Nrdp1 'preferentially' promotes TLR-mediated production of type I interferon. *Nat. Immunol.* **10**:744–752.
 51. **Wang, D., et al.** 2010. Foot-and-mouth disease virus leader proteinase inhibits dsRNA-induced type I interferon transcription by decreasing interferon regulatory factor 3/7 in protein levels. *Biochem. Biophys. Res. Commun.* **399**:72–78.
 52. **Wertz, I. E., et al.** 2004. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature* **430**:694–699.
 53. **Wilkinson, K. D., et al.** 1995. Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. *Biochemistry* **34**:14535–14546.
 54. **Zheng, D., G. Chen, B. Guo, G. Cheng, and H. Tang.** 2008. PLP2, a potent deubiquitinase from murine hepatitis virus, strongly inhibits cellular type I interferon production. *Cell Res.* **18**:1105–1113.