

The papain-like protease of avian infectious bronchitis virus has deubiquitinating activity

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Abstract Coronavirus papain-like proteases (PLPs) can act as proteases that process virus-encoded large replicase polyproteins and also as deubiquitinating (DUB) enzymes. Like the PLPs of other coronaviruses (CoVs), the avian infectious bronchitis virus (IBV) PLP catalyzes proteolysis of Gly-Gly dipeptide bonds to release mature cleavage products. However, the other functions of the IBV PLP are not well understood. In this study, we found that IBV exhibits strong global DUB activity with significant reductions of the levels of ubiquitin (Ub)-, K48-, and K63-conjugated proteins. The DUB activity exhibited a clear time dependence, with stronger DUB activity in the early stage of viral infection. Furthermore, the IBV replicase-encoded PLP, including the downstream transmembrane (TM) domain, is a DUB enzyme and dramatically reduced the level of Ub-conjugated proteins, while processing both K48- and K63-linked polyubiquitin chains. By contrast, PLP did not cause any reduction of haemagglutinin (HA)-Ub-conjugated proteins. In addition, mutations of the catalytic residues of PLP-TM, Cys1274Ser and His1437Lys, reduced DUB activity against Ub-, K48- and K63-conjugated proteins, indicating that the DUB activity of the PLP-TM wild-type protein is not completely dependent on its catalytic activity. Overall, these results demonstrate that the IBV-encoded PLP-TM functions as a DUB enzyme and suggest that IBV may interfere with the activation of host

antiviral signaling pathway by degrading polyubiquitin-associated proteins.

Introduction

Avian infectious bronchitis virus (IBV) is the prototype of gamma coronaviruses (CoV), a family of enveloped viruses that possess a large continuous positive-stranded RNA genome [4]. The genomic RNA is 27.6 kb in length, and approximately two thirds of the nucleotide sequence encodes ORF 1, which includes ORF 1a and ORF 1b [11, 22–25]. A papain-like protease (PLP) is encoded by the region from nucleotides 4243 to 5553 of ORF 1a [21], comprising the catalytic domain of nonstructural protein 3 (nsp3). Proteolysis at the Gly673-Gly674 and Gly2265-Gly2266 dipeptide bonds leads to release of the 87-kDa and 195-kDa N-terminal mature proteins and the C-terminal 41-kDa cleavage product [20, 21, 23]. Site-directed mutagenesis studies have confirmed that the Cys1274 and His1437 residues of PLP are essential for proteinase activity [20].

CoVs such as the human coronavirus NL63 (HCoV-NL63) [6] and murine hepatitis virus-A59 (MHV-A59) [2] encode two functional papain-like proteases (PLP), termed PLP1 and PLP2. At least two CoVs, severe acute respiratory syndrome coronavirus (SARS-CoV) [1] and IBV [21] encode only one functional papain-like protease, termed PLP. The proteolytic processing mediated by the PLP encoded by IBV [20] is similar to that of PLP2 encoded by other CoVs [3, 6]. PLPs of CoVs also perform deubiquitinating (DUB) and interferon (IFN) antagonism activities. For instance, PLP2 of MHV-A59 [29] can bind to IRF3, cause its deubiquitination and prevent its nuclear translocation, thus inhibiting cellular IRF3-mediated type I IFN

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production and promoting viral infection. The SARS-CoV PLP pro-catalytic core also has DUB activity and can deubiquitinate IRF3, thereby stalling its migration to the nucleus and preventing the host antiviral response [7, 10, 26]. Similarly, purified PLP2 of HCoV-NL63 can hydrolyze K48-linked hexa-ubiquitin (K48-Ub6) to produce monoubiquitin [6, 7], and therefore negatively regulate antiviral defenses by disrupting the STING-mediated IFN induction [26].

Structural and enzymatic studies have revealed that CoVs PLPs can act as both a protease, to process virus-encoded large replicase polyproteins, and a DUB enzyme, to cleave the isopeptide bonds found in polyubiquitin chains [7, 20]. Here, we demonstrate that IBV has DUB activity, and like other CoVs, IBV can recognize and process both K48- and K63-linked polyubiquitin chains. We also demonstrate that the core domain of IBV PLP-TM is a coronaviral DUB enzyme. We also evaluated the role of PLP catalytic activity in DUB activity and found that PLP-TM does not require catalytic activity to cleave polyubiquitin-linked proteins. This study represents a first step in elucidating the role of PLP-TM in IBV pathogenesis and provides new insights on how IBV escapes host antiviral immune mechanisms.

Materials and methods

Cells and virus

Chicken embryonic kidney (CEK) cells were aseptically generated from 20-day-old specific pathogen-free (SPF) chicken embryos (Beijing Merial Vital Laboratory Animal Technology Company). The cell suspension was obtained by trypsinization of kidneys for 30 min at 37 °C and subsequent filtration through a 100- μ m mesh. The cells were then cultured in M199 medium (Hyclone) supplemented with 3% fetal bovine serum (FBS). The DF1 chicken fibroblast cell line was used for all transfection-based assays. The cells

were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% FBS.

IBV (JS/2010/12 strain, GenBank accession no. JQ900122.1) was cultured in 10-day SPF chicken embryos or CEK cells. The initial IBV stock was inoculated in chicken embryos for six passages (P6). This study was conducted based on this P6 stock of IBV. The 50% tissue culture infective dose (TCID₅₀) of the IBV P6 stock was determined by identifying the cytopathic effect (CPE) of the virus in CEK cells.

Plasmids

The plasmid pcDNA-5' flag was a kind gift from Dr. Meng (Dalian Medical University). pRK5-HA-Ub and the mutant derivatives pRK5-HA-Ub-K48 and pRK5-HA-Ub-K63 were obtained from Addgene (plasmids #17608, #17605 and #17606) [19]. Attachment of ubiquitin (Ub) modifiers is a reversible post-translational modification that regulates the fate and function of proteins. The ubiquitin molecule contains a total of seven lysine residues at positions 6, 11, 27, 29, 33, 48 (K48), and 63 (K63). These lysine residues potentially mediate ubiquitin chain elongation. The two most common types of polyubiquitin chains are linked through ubiquitin lysine 48 (K48) and lysine 63 (K63).

Generation of the IBV PLP and PLP-TM constructs and site-directed mutants

PLP is encoded by IBV ORF 1a at the region of nsp3 between nucleotides 4243 and 5553 [22]. Biological analysis software (TMHMM Server v. 2.0) was used to analyze the amino acid sequence of nsp3 to predict the transmembrane (TM) domain near PLP [9]. In Figure 2b, the red area indicates the transmembrane domain; the extracellular protein and short cytoplasmic domain are indicated by pink and blue, respectively (Fig. 2b).

PLP and PLP-TM constructs were generated using specific primers (Table 1) to amplify the designated

Table 1 Primers used for the amplification or mutagenesis of IBV sequences

Purpose	Name	Sequence (5' → 3')	Position
Cloning of PLP	PLP:F	<u>AGGATCC</u> GGATTGGATGCGCAAAGTAT (<i>Bam</i> H I)	4243-4266 bp
	PLP:R	A <u>ACTCGAGA</u> AACATTGTGTAGTAACAACACTAGTTCC (<i>Xho</i> I)	5527-5553 bp
Cloning of PLP-TM	PLP:F	<u>AGGATCC</u> GGATTGGATGCGCAAAGTAT (<i>Bam</i> H I)	4243-4266 bp
	PLP-TM:R	AA <u>GCGGCCGC</u> ATAAAAAAGCCAGAAATAAAATCCT (<i>Not</i> I)	6228-6252 bp
PLP-TM C1274S	PLP C1274S:F	AAATGGCGTGATGGAAAC <u>a</u> GCTGGATTAGTTTCAG	4330-4363 bp
	PLP C1274S:R	<u>t</u> GTTTCCATCACGCCATTTAAGTAAGATAA	4319-4347 bp
PLP-TM H1437K	PLP H1437K:F	GGTCCACCAACAGTGGAA <u>a</u> TGTTATACACAA	4820-4851 bp
	PLP H1437K:R	<u>t</u> TtTCCACTGTTGGTGGACCCAATAAAAAC	

a. The underlined nucleotides are restriction enzyme sequences and mutated sequences. Restriction enzymes are indicated in parentheses

b. All nucleotide numbers are based on the IBV JS/2010/12 strain genome sequence

regions from IBV cDNA by PCR. The amplified products of PLP (nucleotides 4243 to 5553) were cloned into pcDNA-5' flag at the *Bam*HI and *Xho*I sites as an in-frame fusion with the flag peptide. PLP, including the TM domain (nucleotides 4243 to 6252), was cloned into pcDNA-5' flag at the *Bam*HI and *Not*I sites to generate TM-containing PLP (PLP-TM) in frame with the flag peptide.

A cysteine residue (Cys 1274) and histidine residue (His 1437) of PLP are the catalytic residues of the proteinase activity [20]. Either of these two sites could play an important role in the DUB activity of PLP-TM. To explore these possibilities, substitution mutations of the Cys 1274 residue with Ser and the His 1437 residue with Lys were constructed to obtain the mutant constructs PLP-TM Cys1274Ser (PLP-TM C1274S) and PLP-TM His1437Lys (PLP-TM H1437K) [20]. To generate specific mutations of the catalytic residues, mutagenic primers (Table 1) were incorporated into newly synthesized DNA using the Fast mutagenesis system protocol (Trans, Fast Mutagenesis System, FM111) according to the manufacturer's instructions.

Transfection

The transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. This method resulted in a transfection rate of at least 70%. Briefly, the plasmid (2 µg for 6-well) was diluted into opti-MEM, and Lipofectamine 2000 (5 µl for 6-well) was diluted into opti-MEM. The diluted DNA was added to diluted Lipofectamine 2000 (1:1 ratio), incubated for 5 min, and then added to the cell cultures.

Western blot

DF1 cells were co-transfected with pRK5-HA-Ub, pRK5-HA-Ub-K48 or pRK5-HA-Ub-K63 plasmids and the indicated amounts of IBV PLP, PLP-TM and specific catalytic mutants. The cells were then lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, China). The cell lysates were analyzed for HA-conjugated proteins using western blotting with monoclonal anti-HA antibody (1:5000, Sigma). An anti-N antibody was used to detect the viral replication level. To confirm the expression levels of PLP and the mutants, anti-flag antibody (1:5000, Sigma) was used to detect the flag-tagged proteins. Actin was detected using β-actin antibody (1:5000, Sigma) as a protein loading control.

IBV DUB activity detection assays

The effect of IBV on ubiquitinated proteins in cells was assessed as follows. DF1 cells were infected with IBV at a multiplicity of infection (MOI) of 10 or mock-infected. The

cells were then transfected with pRK5-HA-Ub, pRK5-HA-Ub-K48 or pRK5-HA-Ub-K63 for an additional 24 h. To investigate the relationship between IBV replication and DUB activity, DF1 cells were infected with IBV at an MOI of 10 or mock-infected and then transfected with pRK5-HA-Ub at 12 h, 18 h or 24 h post-infection for an additional 24 h. Cell lysates were then prepared and the extent of protein ubiquitination was assessed by western blot as described.

Assays for the DUB activity of PLP, PLP-TM and the catalytic mutants

The effects of PLP, PLP-TM and the catalytic mutants on ubiquitinated proteins in cultured cells was assessed as follows. DF1 cells were co-transfected with pRK5-HA-Ub/pRK5-HA-Ub-K48/pRK5-HA-Ub-K63 and the indicated amounts of the constructs encoding PLP-TM or the corresponding catalytic mutants. The pcDNA-5' flag vector was used to standardize the quantity of DNA. The total cells were harvested, and ubiquitinated proteins were assessed as described previously 24 h post infection.

Results

IBV has DUB activity

To determine if IBV exhibits DUB activity against host cellular substrates after infection, DF1 cells were transfected with pRK5-HA-Ub, pRK5-HA-Ub-K48 or pRK5-HA-Ub-K63 after infection with IBV or mock infection. The levels of Ub- (Fig. 1a, lane 3), K48- (Fig. 1a, lane 5) and K63- (Fig. 1a, lane 7) conjugated proteins were reduced dramatically in DF1 cells after infection with IBV. This suggests that IBV exhibits strong DUB activity that recognizes and processes both K48- and K63-linked polyubiquitin chains. To investigate the relationship between IBV replication and DUB activity, DF1 cells were transfected with pRK5-HA-Ub at different times post-infection. Western blot analysis revealed that IBV decreased the levels of Ub-conjugated proteins at different times, and the levels of Ub-conjugated proteins were lower at 12 h than at 18 h and 24 h. Thus, IBV exhibited stronger DUB activity in the early stage of infection (Fig. 1b, lanes 3, 4 and 5; Fig. 1c). Overall, IBV has global DUB activity against ubiquitinated proteins, and the DUB activity exhibits a clear time dependence in cultured cells.

PLP-TM, but not PLP, has DUB activity

PLP is the catalytic domain of IBV, encoded by the genomic region from nucleotides 4243 to 5553, and the TM

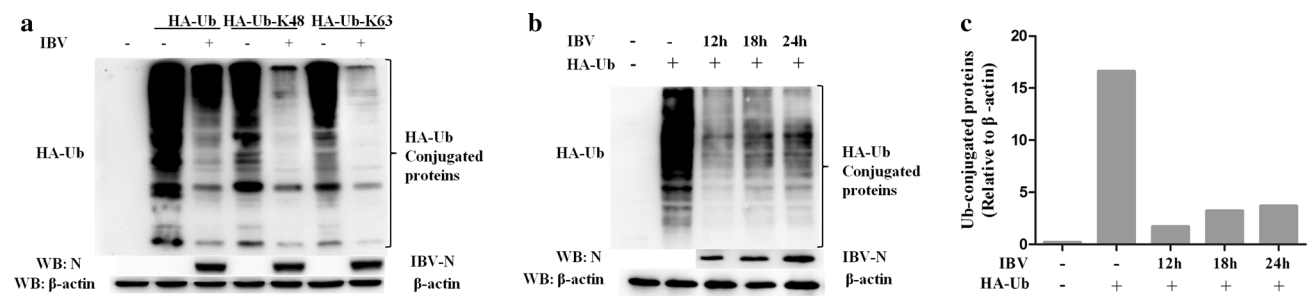


Fig. 1 IBV has DUB activity against Ub- and K48- and K63-linked polyubiquitinated proteins, and DUB activity is stronger in the early stage of viral infection. (a) DUB activity of IBV for Ub- and K48- and K63-linked polyubiquitin. DF1 cells were transfected with HA-tagged ubiquitin and then infected with IBV at an MOI of 10 or mock-infected. (b) DF1 cells were infected with IBV at an MOI of 10 or mock-infected and then the infected cells were transfected with pRK5-HA-Ub at 12 h, 18 h or 24 h for an additional 24 h. The HA-

tagged Ub-conjugated protein was assayed with an anti-HA antibody by western blot (top panel). β -actin was detected as a loading control (middle panel). The IBV-encoded N protein was detected with an anti-N antibody (bottom panel). (c) The Ub-conjugated proteins in Fig. 1b were quantified using biological analysis software (Image-Pro-plus 6.0). The fold change of Ub-conjugated proteins was expressed as densitometric units of bands normalized to β -actin

domain is encoded by the region from nucleotides 5781 to 6252, downstream of PLP (Fig. 2a, b). To determine if PLP and PLP-TM have DUB activity, PLP and PLP-TM were separately co-transfected with pRK5-HA-Ub into DF1 cells. We found that expression of PLP-TM resulted in a dramatic reduction in the level of Ub-conjugated proteins (Fig. 2c, lane 3). By contrast, expression of PLP did not result in any significant reduction of HA-Ub conjugates (Fig. 2c, lane 4). This result indicates that IBV PLP-TM has effective DUB activity that can remove Ub conjugates from many cellular substrate proteins.

PLP-TM removes both K48- and K63-linked polyubiquitin chains

The two most common types of ubiquitinated proteins are linked through ubiquitin K48 and K63. To determine if PLP-TM of IBV has selectivity for ubiquitinated substrates with K48 and K63 linkages, DF1 cells were transfected with PLP-TM with or without pRK5-HA-Ub (Fig. 2d, lanes 2 and 3), pRK5-HA-Ub-K48 (Fig. 2d, lanes 4 and 5) or pRK5-HA-Ub-K63 (Fig. 2d, lanes 6 and 7). The extent of ubiquitinated products was assessed by western blot. Consistent with the processing of Ub-linked proteins by PLP-TM, PLP-TM dramatically reduced the levels of both K48- and K63-linked ubiquitin (Fig. 2b, lanes 3, 5 and 7). This result shows that both major forms of polyubiquitinated proteins can be recognized and degraded by PLP-TM.

The DUB activity of PLP-TM does not completely depend on its catalytic activity

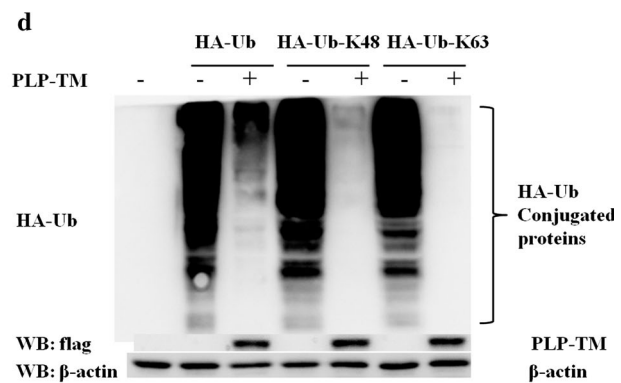
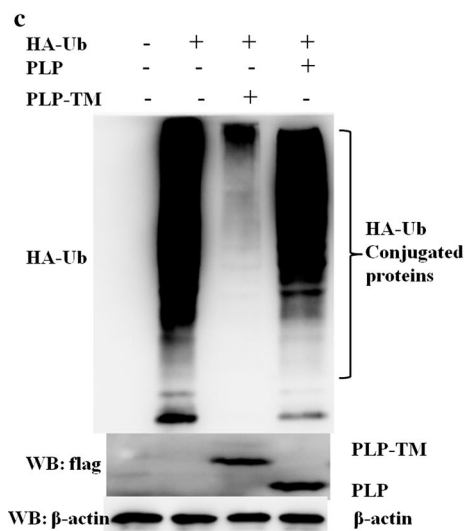
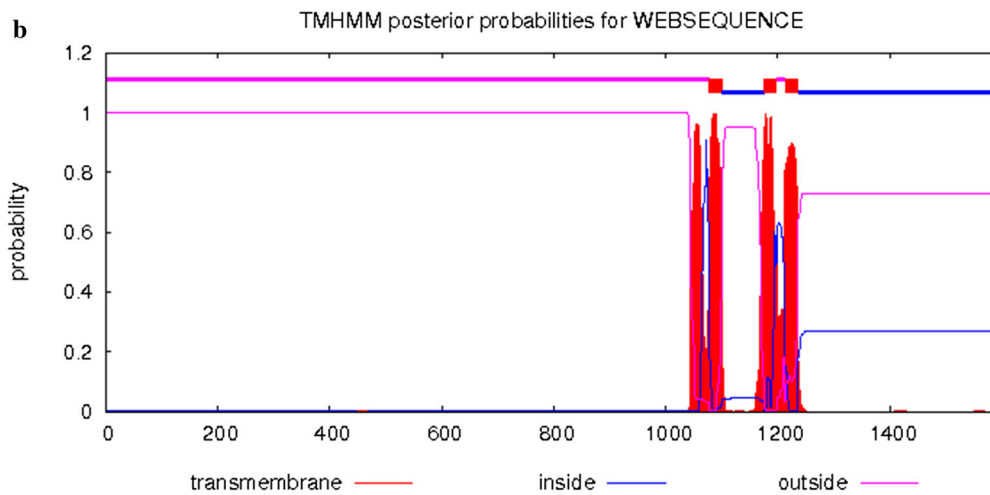
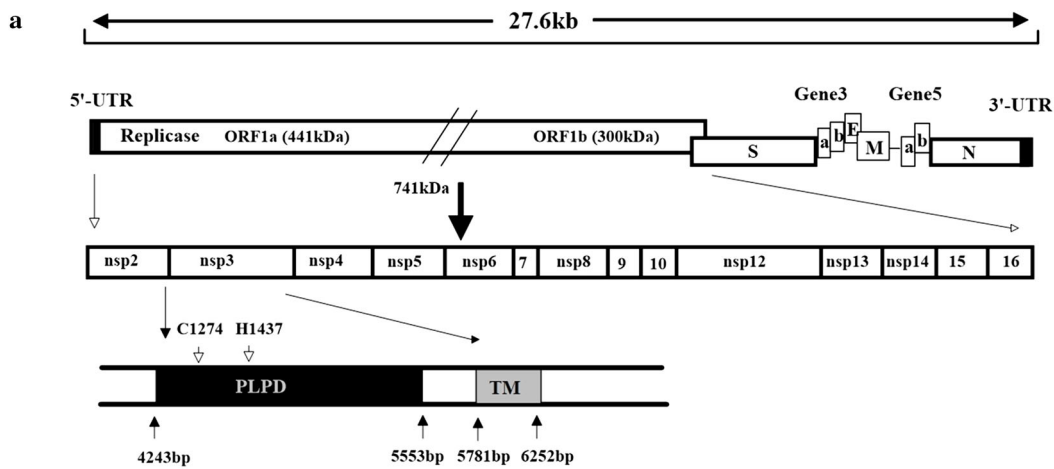
PLP contained within the nsp3 has been shown to be responsible for the cleavage of ORF 1a at the two Gly-Gly dipeptide bonds to release mature protein, and the catalytic dyad for this activity was Cys1274 and His1437 [20].

Fig. 2 IBV PLP-TM, but not PLP, has global deubiquitinating activity in cultured cells. (a) Schematic diagram of the IBV genomic RNA and the resulting polyprotein 1ab, which includes the viral protease PLP. The flag-tagged constructs of PLP used in this study are listed and the catalytic residues numbers from ORF 1a are shown [9, 21]. (b) Prediction of the transmembrane helical domain near PLP. The transmembrane domain (red) is identified with high probability; the predicted topology suggests that most of the protein is extracellular (pink) with a short cytoplasmic domain (blue). (c) PLP and PLP-TM were transfected into DF1 cells along with pRK5-HA-Ub. (d) DF1 cells were co-transfected with pRK5-HA-Ub or pRK5-HA-Ub-K48 or pRK5-HA-Ub-K63, along with the indicated amounts of PLP-TM. Cell lysates were prepared at 24 h post-transfection and analyzed for HA-Ub-conjugated proteins by western blot with an anti-HA antibody (top panel). Anti-flag was used to confirm the expression of PLP and PLP-TM (second panel), and β -actin was detected as a loading control (bottom panel) (color figure online)

Either of the two catalytic residue sites may play an important role in the DUB activity of PLP-TM. To explore these possibilities, DF1 cells were transfected with PLP-TM, PLP-TM C1274S or PLP-TM H1437K, together with pRK5-HA-Ub (Fig. 3a), pRK5-HA-Ub-K48 (Fig. 3b), or pRK5-HA-Ub-K63 (Fig. 3c), and the extent of ubiquitinated products was assessed by western blot. We found that the catalytic mutants (C1274S and H1437K) of PLP-TM exhibited reduced DUB activity against Ub-, K48- and K63-linked proteins (Fig. 3a–c). These results demonstrate that the DUB activity of PLP-TM is not completely dependent on its catalytic activity.

Discussion

In this study, we characterized the DUB activity of IBV PLP in DF1 cells and the core domain of the DUB activity. We found that IBV exhibits strong global DUB activity during infection of DF1 cells, indicating that IBV infection disrupts polyubiquitin modification in host cells or encodes



a protein with DUB activity [14]. Further experiments indicated that the proteinase PLP-TM plays an important role in IBV DUB activity and can process both

K63-linked polyubiquitin chains. Moreover, the DUB activity of PLP-TM was not completely dependent on its catalytic activity.

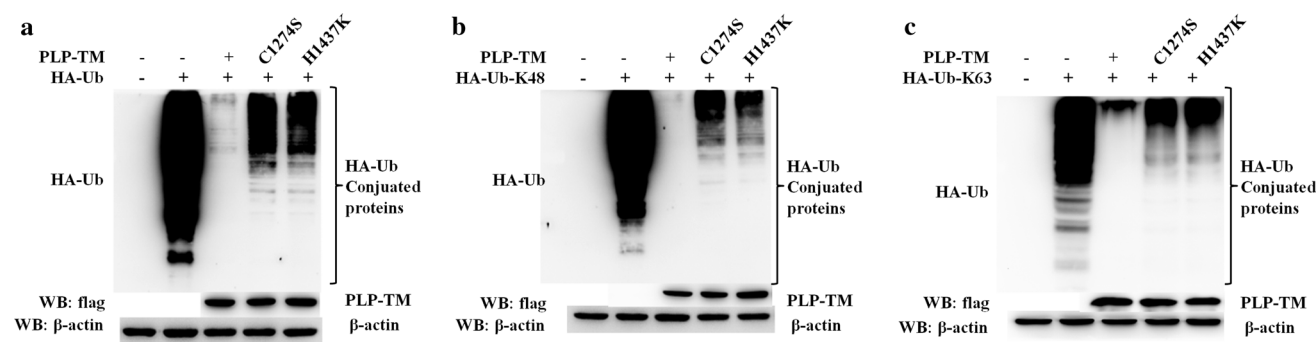


Fig. 3 The DUB activity of PLP-TM is not completely dependent on its catalytic activity. IBV PLP-TM, PLP-TM C1274S and PLP-TM H1437K were transfected into DF1 cells along with pRK5-HA-Ub (a), pRK5-HA-Ub-K48 (b) or pRK5-HA-Ub-K63 (c). The cell lysates were prepared at 24 h post-transfection and analyzed for HA-Ub-

conjugated proteins by western blot with an anti-HA antibody (top panel). Anti-flag was used to confirm the expression of PLP-TM and the catalytic mutants (second panel), and β -actin was detected as a loading control (bottom panel)

Similar to the CoV porcine epidemic diarrhea virus (PEDV) [28], the DUB activity of IBV also dramatically reduced Ub-conjugated protein levels in virus-infected cells, and the IBV PLP plays an important role in this DUB activity. PLP2 of PEDV has potent DUB activity that is dependent on its catalytic activity. In notable contrast to PEDV PLP2, the DUB activity of IBV PLP is not dependent on its protease activity. Another group reported that IBV PLP can degrade K48- and K63-linked polyubiquitin chains to monoubiquitin but cannot degrade linear polyubiquitin [18]. HCoV-NL63 PLP2 has a DUB activity that is dependent on its protease activity [7]. By contrast, both the PLP2-TM and catalytic mutants of PLP2-TM [26] had DUB activity, although the DUB activity of the PLP2-TM catalytic mutants was lower than that of PLP2-TM, suggesting that the TM domain plays a role in PLP2 DUB activity. Similarly, our study confirmed that the TM domain downstream of IBV PLP is essential for PLP DUB activity. The IBV PLP cleaves the ORF 1a to generate nonstructural proteins that associate with endoplasmic reticulum membranes to generate convoluted membranes and double membrane vesicles (DMVs), which are the site of viral replication. The PLPs are tethered to the DMVs by a TM domain [12, 17]. Many reports have demonstrated that the TM domain plays an important role in CoVs DUB activity, possibly by facilitating correct protein folding or interactions with cellular proteins. Additional studies are needed to identify the exact functions of the TM domain during IBV replication and the interaction between the host innate immune system and IBV infection.

This study raises important questions concerning the role of viral DUB activity in CoV replication and pathogenesis. Coronaviral DUB activity may target the Ub-proteasome pathway to facilitate virus replication and damage host defense mechanisms, including innate immunity [5, 8]. Some CoVs may utilize DUB activity to escape the host innate antiviral response [13, 27]. For

example, PLP2 of MHV-A59 can bind to IRF3, cause its deubiquitination and prevent its nuclear translocation. Co-expression of PLP2 inhibits CARDIF-, TBK1- and IRF3-mediated IFN- β reporter activities [29]. MHV-A59 may use DUB activity to reduce IFN induction, to promote viral growth and to escape from host innate antiviral responses [29]. PEDV infection suppresses the production of IFN- β and PLP2 acts as a viral DUB to interfere with RIG-I- and STING-mediated signaling pathways [28]. However, some CoVs PLP-mediated interferon antagonism is independent on protease and DUB activity. The PLP2 of NL63 has DUB activity and antagonizes the induction of type I IFN, whereas PLP-mediated IFN antagonism is independent of DUB activity [6]. These data show that CoVs PLPs target the activity of type I IFN through DUB activity and inhibit the activation of the innate immune system. Joeri Kint et al. showed that IBV inhibits the synthesis of host proteins, including type I IFN, a key component of the antiviral response [15, 16]. Our study also indicates that IBV PLP-TM process both K48- and K63-linked ubiquitin. K48- and K63-linked ubiquitin are the most common types of modified polyubiquitin and play key roles in protein degradation and in response to changes in the innate and adaptive immunity systems. Further investigations are necessary to determine if IBV PLP DUB activity inhibits the host antiviral response.

Overall, the results of our study show that IBV has DUB activity and confirm that PLP-TM is not only a classic papain-like protease encoded by IBV but is also a multifunctional protein that plays important roles in the regulation of interactions between IBV and host antiviral innate immune response proteins. Ubiquitin modifications play key regulatory roles in protein degradation and in innate and adaptive immunity signaling pathways. IBV PLP-TM may prevent the activation of host antiviral signaling pathways by degrading polyubiquitin chains associated with ubiquitin proteins. The interactions between IBV and

the host must be clarified to provide further insight into viral replication and pathogenesis.

Compliance with ethical standards

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Conflict of interest We have no conflict of interest.

Ethical approval The Jiangsu Administrative Committee for Laboratory Animals approved all animal studies (Permit Number: SYXKSU-2007-0005) according to the guidelines of Jiangsu Laboratory Animal Welfare and Ethical of Jiangsu Administrative Committee of Laboratory Animals.

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