

Proteolytic processing, deubiquitinase and interferon antagonist activities of Middle East respiratory syndrome coronavirus papain-like protease

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The emerging Middle East respiratory syndrome coronavirus (MERS-CoV) causes severe pulmonary disease in humans and represents the second example of a highly pathogenic coronavirus (CoV) following severe acute respiratory syndrome coronavirus (SARS-CoV). Genomic studies revealed that two viral proteases, papain-like protease (PLpro) and 3C-like protease (3CLpro), process the polyproteins encoded by the MERS-CoV genomic RNA. We previously reported that SARS-CoV PLpro acts as both deubiquitinase (DUB) and IFN antagonist, but the function of the MERS-CoV PLpro was poorly understood. In this study, we characterized MERS-CoV PLpro, which is a protease and can recognize and process the cleavage sites (CS) of nsp1-2, nsp2-3 and nsp3-4. The LXGG consensus cleavage sites in the N terminus of pp1a/1ab, which is generally essential for CoV PLpro-mediated processing, were also characterized in MERS-CoV. MERS-CoV PLpro, like human SARS-CoV PLpro and NL63-CoV PLP2, is a viral deubiquitinating enzyme. It acts on both K48- and K63-linked ubiquitination and ISG15-linked ISGylation. We confirmed that MERS-CoV PLpro acts as an IFN antagonist through blocking the phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF3). These findings indicate that MERS-CoV PLpro acts as a viral DUB and suppresses production of IFN- β by an interfering IRF3-mediated signalling pathway, in addition to recognizing and processing the CS at the N terminus of replicase polyprotein to release the non-structural proteins. The characterization of proteolytic processing, DUB and IFN antagonist activities of MERS-CoV PLpro would reveal the interactions between MERS-CoV and its host, and be applicable to develop strategies targeting PLpro for the effective control of MERS-CoV infection.

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INTRODUCTION

In September 2012, a new human coronavirus (CoV) emerged in Jeddah and Saudi Arabia and quickly spread to some European countries (Anderson & Baric, 2012; Chan *et al.*, 2012; Zaki *et al.*, 2012). This new CoV, named Middle East respiratory syndrome coronavirus (MERS-CoV), was formerly called human coronavirus-Erasmus Medical Center (HCoV-EMC) (de Groot *et al.*, 2013). It can cause severe acute respiratory syndrome and renal failure. From September 2012 to 1 August 2013, MERS-CoV infected at least 94 people with a fatality rate of about

50% globally (novel coronavirus infection update; http://www.who.int/csr/disease/coronavirus_infections/en/index.html). The epidemic pattern and clinical symptoms caused by MERS-CoV are very similar to the severe acute respiratory syndrome coronavirus (SARS-CoV) (Booth & Stewart, 2005; Chan-Yeung & Xu, 2003). Person-to-person transmission of MERS-CoV was recently confirmed; the virus can spread globally and cause considerable morbidity endangering world health and the economy (Assiri *et al.*, 2013).

Several isolates of MERS-CoV have been fully sequenced and confirmed to belong to group C β -coronaviruses (Chan *et al.*, 2012; Khan, 2013; Lau *et al.*, 2013; van Boheemen *et al.*, 2012). The analysis of genome size,

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organization and sequence revealed that MERS-CoV is most closely related to bat CoVs, BtCoV-HKU4 and BtCoV-HKU5 (Chan *et al.*, 2012; Lau *et al.*, 2013; van Boheemen *et al.*, 2012). The MERS-CoV genome is just over 30 kb and contains at least 10 predicted ORFs including polyprotein pp1a/1ab, S protein, E protein, N protein and some canonical structural proteins (van Boheemen *et al.*, 2012). The ORF1a sequence of MERS-CoV encodes two protease domains that are conserved in all other CoVs, a papain-like protease (PLpro) in non-structural protein (nsp) 3 and a 3C-like protease (3CLpro; also known as the ‘main protease’) in nsp5 (van Boheemen *et al.*, 2012). Sequence comparison of MERS-CoV with other CoVs allowed us to predict the putative pp1a/ pp1ab cleavage sites (CS) and annotate the processing products of nsp1-16 (van Boheemen *et al.*, 2012).

Analysis of proteolytic processing of several CoVs has revealed that the replicase polyproteins pp1a and pp1ab are

cotranslationally and post-translationally processed by the viral proteases PLpro and 3CLpro to generate as many as 16 nsps. These nsps contribute to subgenomic RNA synthesis by forming the replicase complex with the endoplasmic reticulum membranes (Chen *et al.*, 2007; Gosert *et al.*, 2002; Prentice *et al.*, 2004; Snijder *et al.*, 2006). Several studies revealed that papain-like protease processes the N terminus of pp1a and pp1ab by processing the CS 1–3 to release nsp1-3, whereas 3CLpro processes the subsequent CS to release nsp4-16 (Barretto *et al.*, 2005; Chen *et al.*, 2007; Oostra *et al.*, 2008). Proteolytic processing of the CoV proteases is essential for correct localization and function of the replicase proteins, ongoing viral RNA synthesis and virus replication (Chen *et al.*, 2007; Kim *et al.*, 1995). In addition to the proteolytic processing, CoV PLpro has deubiquitination activity and serves as an IFN antagonist (Barretto *et al.*, 2005; Clementz *et al.*, 2010;

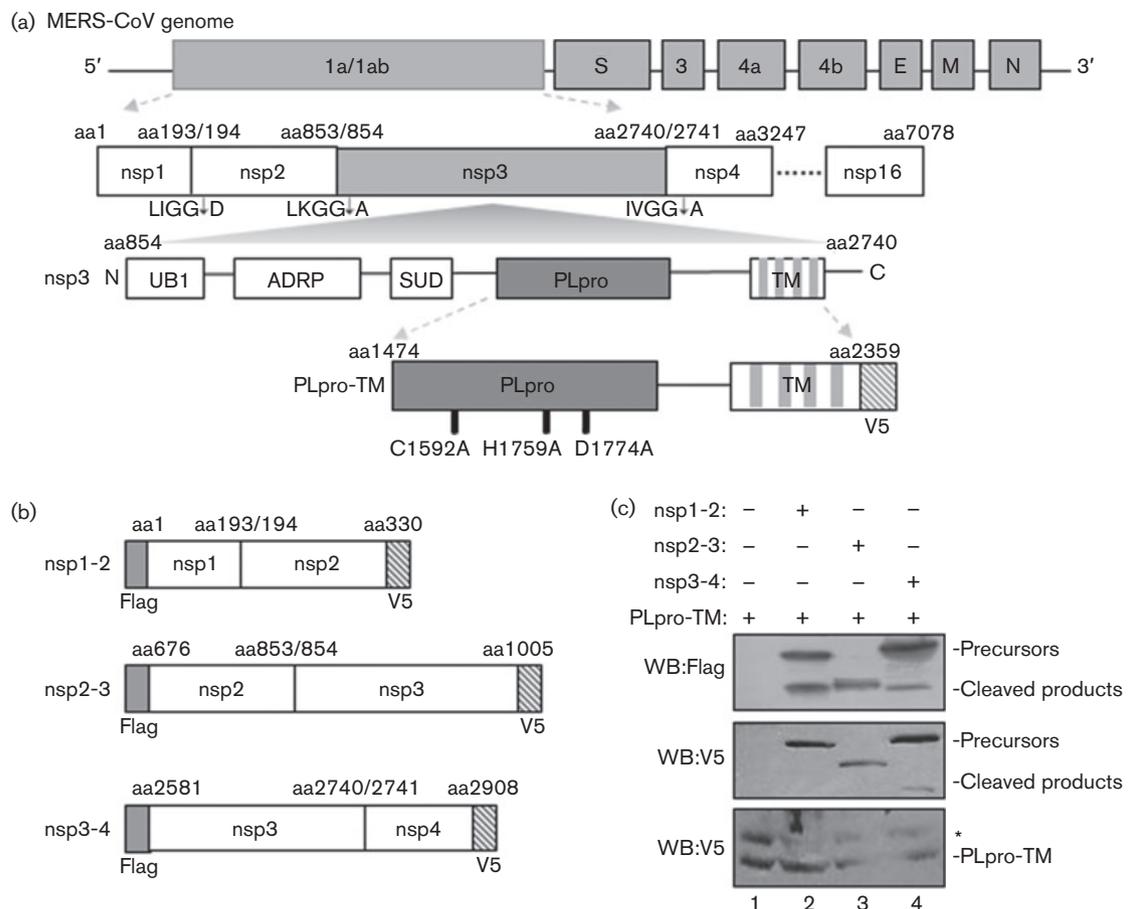


Fig. 1. Identification of proteases that process the MERS-CoV replicase polyprotein. (a) Schematic diagram illustrating MERS-CoV ORFs. The predicted process of replicase polyprotein processing to nsps, and the UB1 (ubiquitin-like fold), ADRP (ADP-ribose-1d-phosphatase), SUD (SARS-unique domain), PLpro and TM domains in nsp3 are indicated. The PLpro-TM construct used in the study is presented. (b) A model illustrating the substrates used in the *trans*-cleavage assay. The constructs nsp1-2, nsp2-3 and nsp3-4 are indicated. (c) MERS-CoV PLpro-TM recognizes and processes the substrates. HEK293T cells were cotransfected with the plasmid DNAs that express PLpro-TM and nsp1-2, nsp2-3 or nsp3-4, and the cells were lysed and subjected to Western blotting with the indicated antibodies to detect the cleavage products. The asterisk indicates the putative glycosylated form of PLpro-TM.

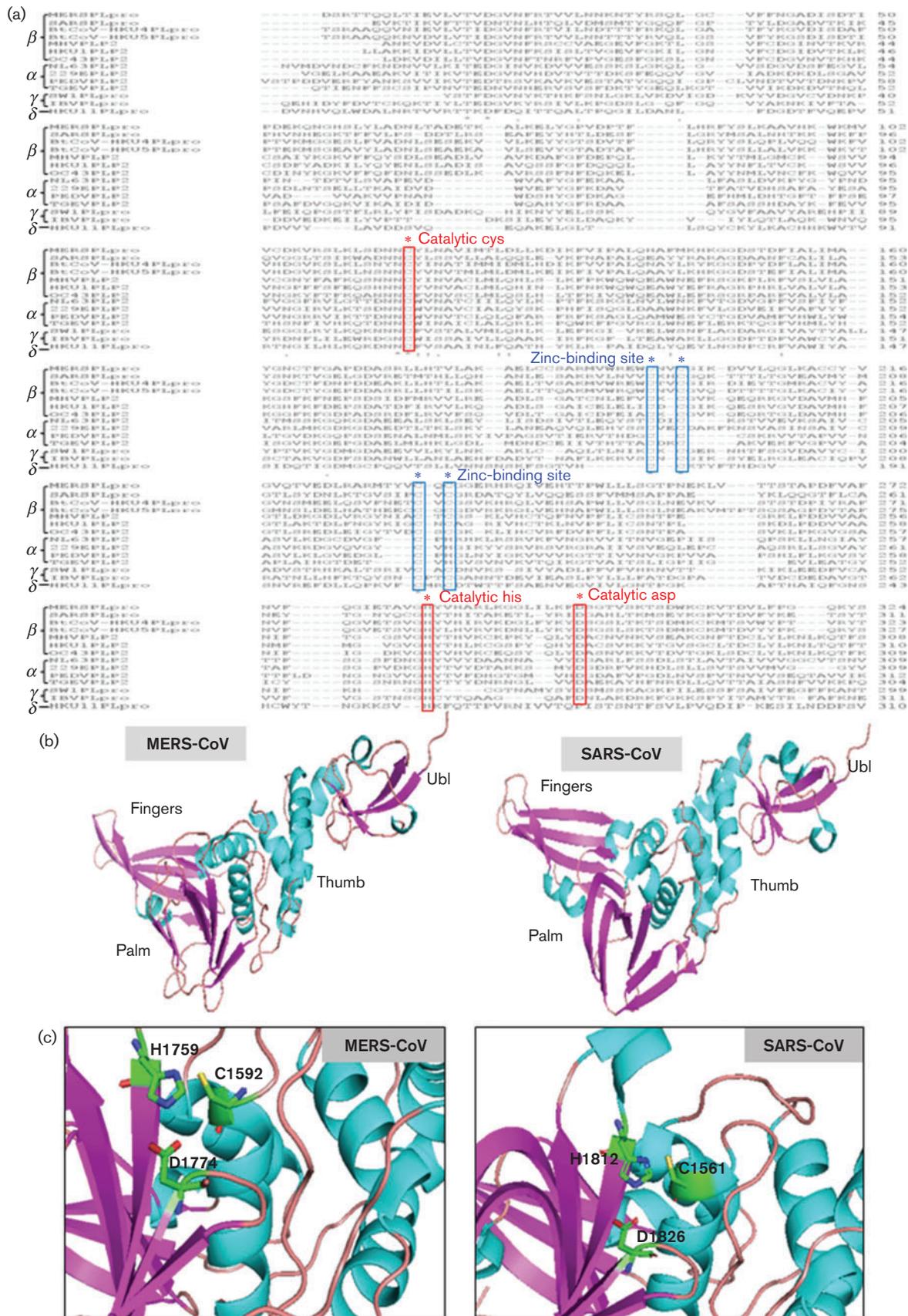


Fig. 2. Multiple sequence alignment and 3D structure model of MERS-CoV papain-like protease domains. (a) Multiple

Fig. 2. (cont.) sequence alignment of CoV PLpro domains. The PLpro domain amino acid sequences of the indicated CoVs were aligned using CLUSTAL 2.1. The amino acids from 1476 to 1804 of MERS-CoV PLpro were used as a reference sequence. Amino acid numbers are indicated on the right. Predicted or experimentally determined catalytic residues (cysteine, histidine and aspartic acid) and the cysteine/histidine residues essential for binding zinc are boxed. Accession numbers are as follows: MERS-CoV: NC_019843.1; SARS-CoV Urbani strain: AY278741; BCoV-HKU4-1 (bat CoV HKU4-1): NC_009019.1; BCoV-HKU5-1: NC_009020.1; HCoV-OC43 (human CoV OC43): NC_005147.1; MHV: NC_001846; HCoV-HKU1: NC_006577.2; HCoV-NL63: NC_005831.2; HCoV-229E: NC_002645.1; PEDV (porcine epidemic diarrhea virus): NC_003436; TGEV (transmissible gastroenteritis virus): Z34093; SW1 (beluga whale CoV SW1, BWCov-SW1): EU111742; IBV: NC_001451; ACoV-HKU11 (avian coronavirus HKU11): FJ376620. (b) 3D structural model of MERS-CoV papain-like protease domains (left). The homology model was built based on the structure of human SARS-CoV PLpro (right) (PDB code, 2FE8) (Ratia *et al.*, 2006) using SWISS-MODEL (Kiefer *et al.*, 2009), an automated protein structure homology-modelling server (<http://beta.swissmodel.expasy.org/workspace>). The Ubl, thumb, palm and fingers domains are indicated in blue, green, red and orange, respectively. The catalytic triad and four zinc-coordinated cysteines are shown as sticks. (c) MERS-CoV PLpro catalytic triad residues, C1592, H1759 and D1774 (left), are indicated in the enlarged 3D structure of PLpro, which is remarkably similar to that of SARS-CoV PLpro (right).

Frieman *et al.*, 2009; Sulea *et al.*, 2005), indicating that PLpro is a multifunctional protease that is involved in CoV replication and modulates the interaction between the virus and its host (Sun *et al.*, 2010, 2012). A very recent study reported the expression and activity of MERS-CoV PLpro and 3CLpro in cell-based biosensor assays, which allows for rapid evaluation of viral protease activity and the identification of protease inhibitors (Kilianski *et al.*, 2013). Thus, CoV proteases are attractive targets for the development of antiviral drugs for reduction of viral replication and pathogenicity. However, the characteristics and function of MERS-CoV PLpro are little known.

In this study, we first identified the MERS-CoV PLpro activity and the putative catalytic triad that is required for cleavage of pp1a/1ab. We found that the membrane-anchored form of PLpro (PLpro-TM) alone is sufficient to process the predicted nsp1/2, nsp2/3 and nsp3/4 sites. The putative cleavage sites between replicase products were also characterized by site-directed mutagenesis of predicted critical residues. Furthermore, we confirmed that, like SARS-CoV PLpro and HCoV-NL63 (Barretto *et al.*, 2005; Chen *et al.*, 2007), MERS-CoV PLpro has deubiquitinase (DUB) activity that recognizes and processes both ubiquitin (Ub) and ISG15-linked conjugations. Finally, MERS-CoV PLpro acts as a potential IFN antagonist through disrupting the phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF3). The identification of PLpro activity and IFN antagonist activity, and characterization of MERS-CoV PLpro DUB would facilitate the comparative studies of CoV protease activity and the development of novel antiviral reagents specifically against HCoV, including MERS-CoV, HCoV-NL63 and SARS-CoV.

RESULTS

Identification of protease activity and the catalytic sites of MERS-CoV PLpro

MERS-CoV has been predicted to encode replicase polyproteins that possess about 35% sequence homology

to those of SARS-CoV (van Boheemen *et al.*, 2012). The MERS-CoV polyproteins were predicted to produce 16 nsps by two distinct proteases, PLpro and 3CLpro (Fig. 1a). Previous studies revealed that expression of an extended region of PLpro/PLP2 that included the downstream transmembrane (TM) domains was required for processing at the predicted N terminal CS in the polyprotein (Harcourt *et al.*, 2004; Kanjanahaluethai *et al.*, 2007). Firstly, in order to identify the protease activity of PLpro, we generated PLpro-TM (Fig. 1a), and three substrate constructs containing the predicted cleavage sites at the N terminus of pp1a/1ab (nsp1-2, nsp2-3 and nsp3-4) (Fig. 1b). A cell-based *trans*-cleavage system was established by coexpressing PLpro-TM with the truncated substrates of MERS-CoV in HEK293T cells. The protease activity was probed by detection of released processing products using anti-Flag antibody or anti-V5 antibody (Fig. 1b, c). The construct PLpro-TM was cotransfected with plasmid DNAs that expressed the substrates of nsp1-2, nsp2-3 or nsp3-4 in HEK293T cells. The cells were harvested and subjected to Western blotting using the indicated antibodies. As shown in Fig. 1c, the WT PLpro completely cleaved the substrate nsp2-3 to produce the cleavage products (Fig. 1c, lane 3). PLpro-TM partially processed the substrates nsp1-2 and nsp3-4, although the processed products were detectable (Fig. 1c, lanes 2 and 4). We note that PLpro-TM is detected as two closely migrating bands, perhaps due to glycosylation of the transmembrane domains, which was confirmed in SARS-CoV and murine hepatitis virus (MHV) (Harcourt *et al.*, 2004; Kanjanahaluethai *et al.*, 2007). These findings indicated that MERS-CoV PLpro has protease activities to process pp1a/1ab at the N terminus to release product nsp1-3, which is consistent with other human CoVs, such as SARS-CoV and NL63-CoV (Barretto *et al.*, 2005; Chen *et al.*, 2007; Harcourt *et al.*, 2004).

To identify which critical amino acid residues within the MERS-CoV PLpro core domain were important for protease activity, we aligned the amino acids of the PLpro core domain (aa1476 to aa1804) of MERS-CoV with PLpro/

PLP2 of representative members from all coronaviral genera (Fig. 2a). We found that the predicted triad catalytic cysteine, histidine and aspartic acid residues (C1592–H1759–D1774), and the cysteine/histidine residues essential for binding zinc were highly conserved; these are marked with red and blue boxes, respectively in Fig. 2(a). The three-dimensional (3D) structure of MERS-CoV PLpro core domain (aa1476 to aa1804) was modelled using bioinformatic analysis, and the results showed that the topology of MERS-CoV PLpro is remarkably similar to that of SARS-CoV PLpro (Fig. 2b). It indicated that MERS-CoV PLpro consists of four distinctive domains: an N-terminal Ubl domain, a palm, a thumb and a finger domain, which form an extended right-hand architecture that closely resembles the structure of SARS-CoV PLpro (Ratia *et al.*, 2006). As shown in Fig. 2(c), the conformation of the predicted catalytic sites (C1592–H1758–D1774) of MERS-CoV PLpro is consistent with the catalytic triads found in SARS-CoV PLpro (Ratia *et al.*, 2006). To further determine if the Cys–His–Asp catalytic triad was critical for protease activity of MERS-CoV PLpro, we performed site-directed mutagenesis and changed the nucleotide sequence to code alanine at the specified sites. A *trans*-cleavage assay was performed by cotransfection of plasmid DNAs expressing WT PLpro-TM and the catalytic mutants C1592A, H1759A and D1774A, with each substrate (nsp1-2, nsp2-3 or nsp3-4), into HEK293T cells. Cells were then lysed and subjected to Western blotting using the indicated antibodies (Fig. 3). We observed that alanine substitution of the Cys–His–Asp catalytic triad of PLpro led to the complete loss of protease activity on the nsp1-2 substrate, whereas the WT PLpro-TM had the protease activity of processing nsp1-2 substrate to release nsp1 (Fig. 3a). Introduction of the mutations to the Cys–His–Asp catalytic triad also abolished the protease activity on the substrates nsp2-3 and nsp3-4 (Fig. 3b, c). However, alanine substitution of the Asp (D1774) in the catalytic triad of PLpro led to only a partial loss of protease activity on the nsp2-3 substrate (Fig. 3b, lane 6). These data indicated that Cys–His–Asp catalytic triad is critical for the maintenance of MERS-CoV PLpro activity.

MERS-CoV PLpro recognizes and cleaves at LXGG consensus cleavage sites

We previously reported that SARS-CoV PLpro was responsible for recognizing the consensus cleavage site, LXGG, which was essential for much CoV PLpro-mediated processing (Barretto *et al.*, 2005; Chen *et al.*, 2007). The consensus CS of MERS-CoV PLpro were compared with those of other human CoVs (Fig. 4a) (Sulea *et al.*, 2006). We found that the predicted CS of MERS-CoV nsp1-2, nsp2-3 and nsp3-4 had consensus LXGG sequences, which are conserved in almost all human CoVs (Kanjanaaluethai *et al.*, 2003; Lim *et al.*, 2000; Sulea *et al.*, 2006; Ziebuhr *et al.*, 2007). In order to confirm whether the LXGG consensus site was important for MERS-CoV PLpro recognition and processing, we constructed mutants at every amino acid position of LXGG using site-directed mutagenesis. To

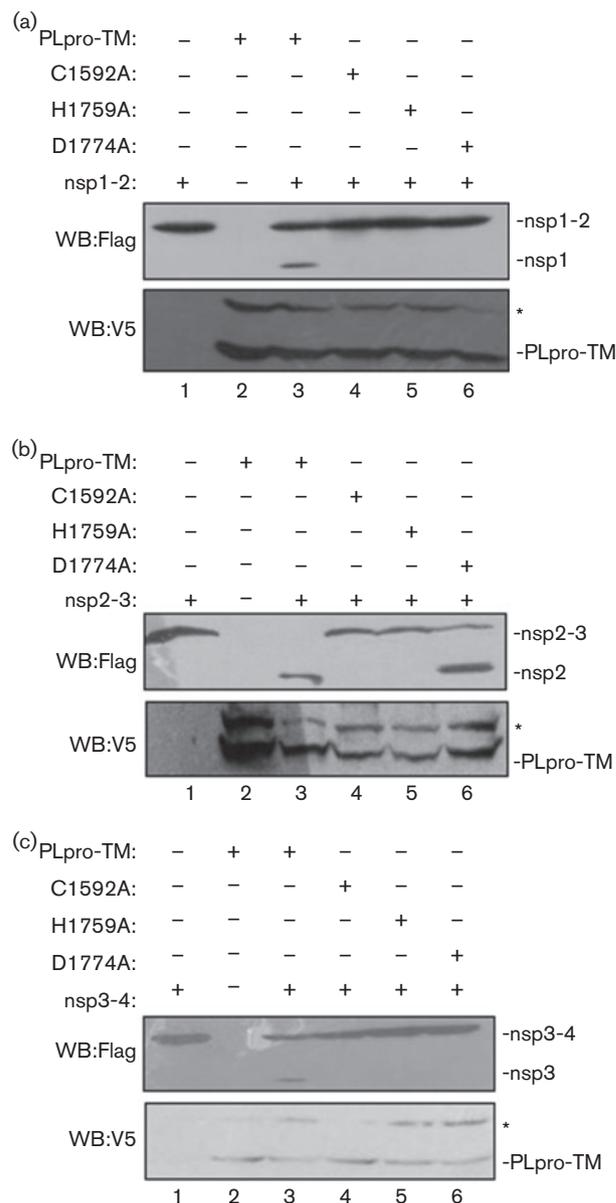


Fig. 3. Identification of residues essential for catalytic activity of MERS-CoV PLpro-TM. HEK293T cells were cotransfected with the substrates nsp1-2 (a), nsp2-3 (b) or nsp3-4 (c) together with PLpro-TM and the catalytic mutants (C1592A, H1759A and D1774A) and the cells were lysed and subjected to Western blotting using the indicated antibodies to detect the cleavage products. The asterisk indicates the putative glycosylated form of PLpro-TM.

examine whether the substrates with each substitution mutation were efficiently processed by PLpro, we cotransfected plasmid DNAs encoding PLpro-TM with WT or the corresponding mutants of the substrates of nsp1-2, nsp2-3 or nsp3-4 into HEK293T cells. The cells were harvested and subjected to Western blotting to detect the processing products as shown in Fig. 4(b–d). The presence of the

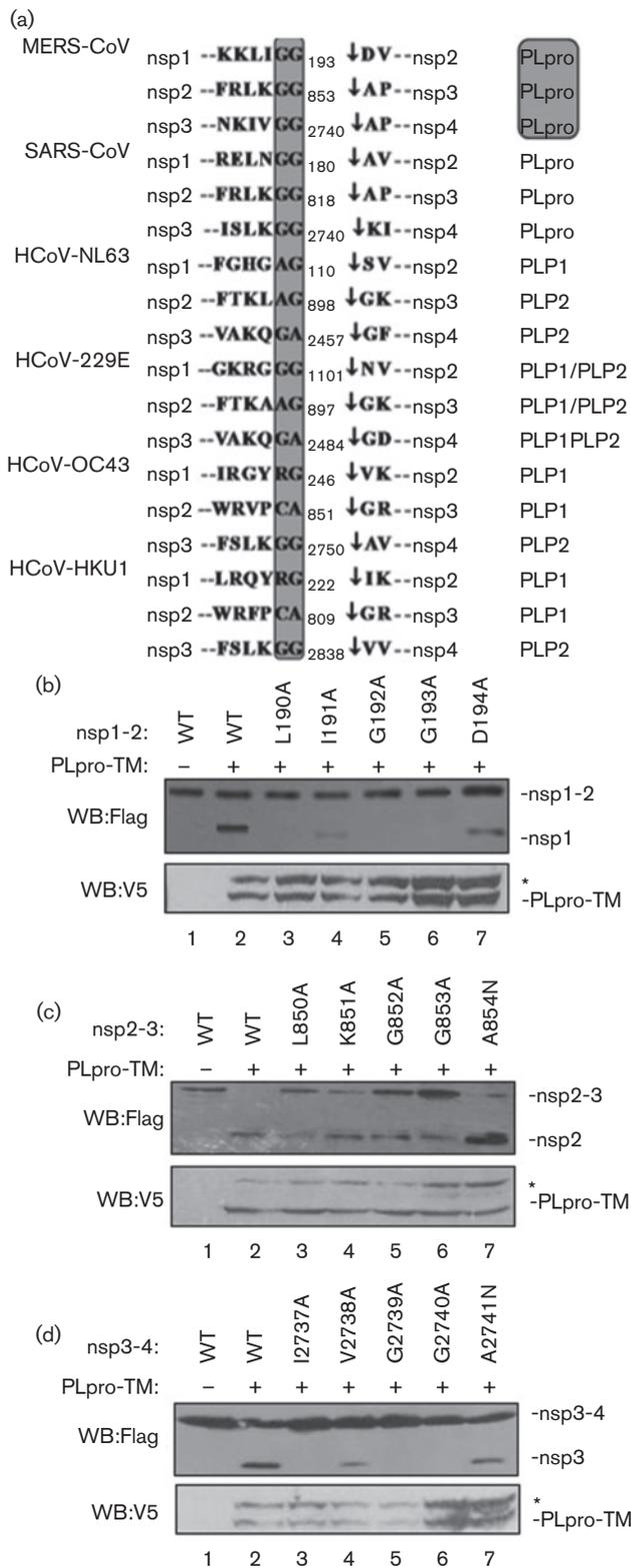


Fig. 4. MERS-CoV PLpro-TM recognizes and processes the consensus sequence LXGG. (a) Assignment of predicted and confirmed cleavage residues in CoV replicase polyproteins. The nsps either side of each cleavage site are indicated. Sites that are highlighted on a grey background are the cleavage sites.

The right column lists the PLpro/PLP responsible for the processing event at each site. Enzyme names are given on the right according to the experimental data. (b, c and d) Alanine or asparagine substitutions were introduced into the predicted cleavage positions of nsp1-2, nsp2-3 and nsp3-4. The plasmid DNAs encoding substrates and the site-directed mutants of nsp1-2 (a), nsp2-3 (b) and nsp3-4 (c) were cotransfected with WT PLpro-TM into HEK293T cells. The cleavage products were detected and analysed by Western blotting using the indicated antibodies. The asterisk indicates the putative glycosylated form of PLpro-TM.

cleavage product indicated that alanine substitution at the site had little or no effect on processing, whereas the absence of the cleavage product indicated that the alanine substitution resulted in a reduction in MERS-CoV PLpro recognition and processing. When mutations were introduced into L190, G192 and G193 of nsp1-2 or I2737, G2739 and G2740 of nsp3-4, PLpro completely lost the processing activity on the substrates nsp1-2 or nsp3-4 (Fig. 4b, d, lanes 3, 5 and 6). Compared with the WT nsp2-3 that was completely cleaved by PLpro, the mutants of all the nsp2-3 substrates were partially processed by PLpro as the substrate precursors were detected, indicating that LKGG at the cleavage site of nsp2-3 is not absolutely required for PLpro recognition and processing (Fig. 4c, lanes 3–6). These findings suggested that the consensus cleavage site LXGG is critical for MERS-CoV PLpro to recognize and process the substrates, although introduction of mutations into the consensus cleavage site LXGG of nsp2-3 significantly reduces the processing efficiency of PLpro.

MERS-CoV PLpro possesses DUB and deISGylation enzyme activities

We and others previously reported that the PLpros of human CoVs, SARS-CoV and NL63-CoV, are coronaviral DUBs (Barretto *et al.*, 2005; Chen *et al.*, 2007; Clementz *et al.*, 2010; Sun *et al.*, 2010). In this study, we examined whether MERS-CoV PLpro exhibited DUB activity against host cellular proteins. If so, what were the roles of PLpro-TM catalytic activity in mediating deubiquitination activity? haemagglutinin (HA)-Ub and either WT or catalytic mutants (C1592A, H1759A and D1774A) of PLpro-TM were transfected into HEK293T cells. The cells were lysed and subjected to Western blotting. A dramatic reduction of Ub-conjugated proteins was detected (Fig. 5a, lane 3). Interestingly, PLpro-TM catalytic mutants (Fig. 3) led to a reduction of Ub-conjugated proteins compared with the control (Fig. 5a, lanes 4, 5 and 6), indicating that MERS-CoV PLpro is a viral DUB and the DUB activity is not dependent on the catalytic activity of PLpro. In addition, we found that WT PLpro-TM and the catalytic mutants led to a significant reduction in the level of both K48- and K63-linked proteins (Fig. 5b, c), indicating that MERS-CoV

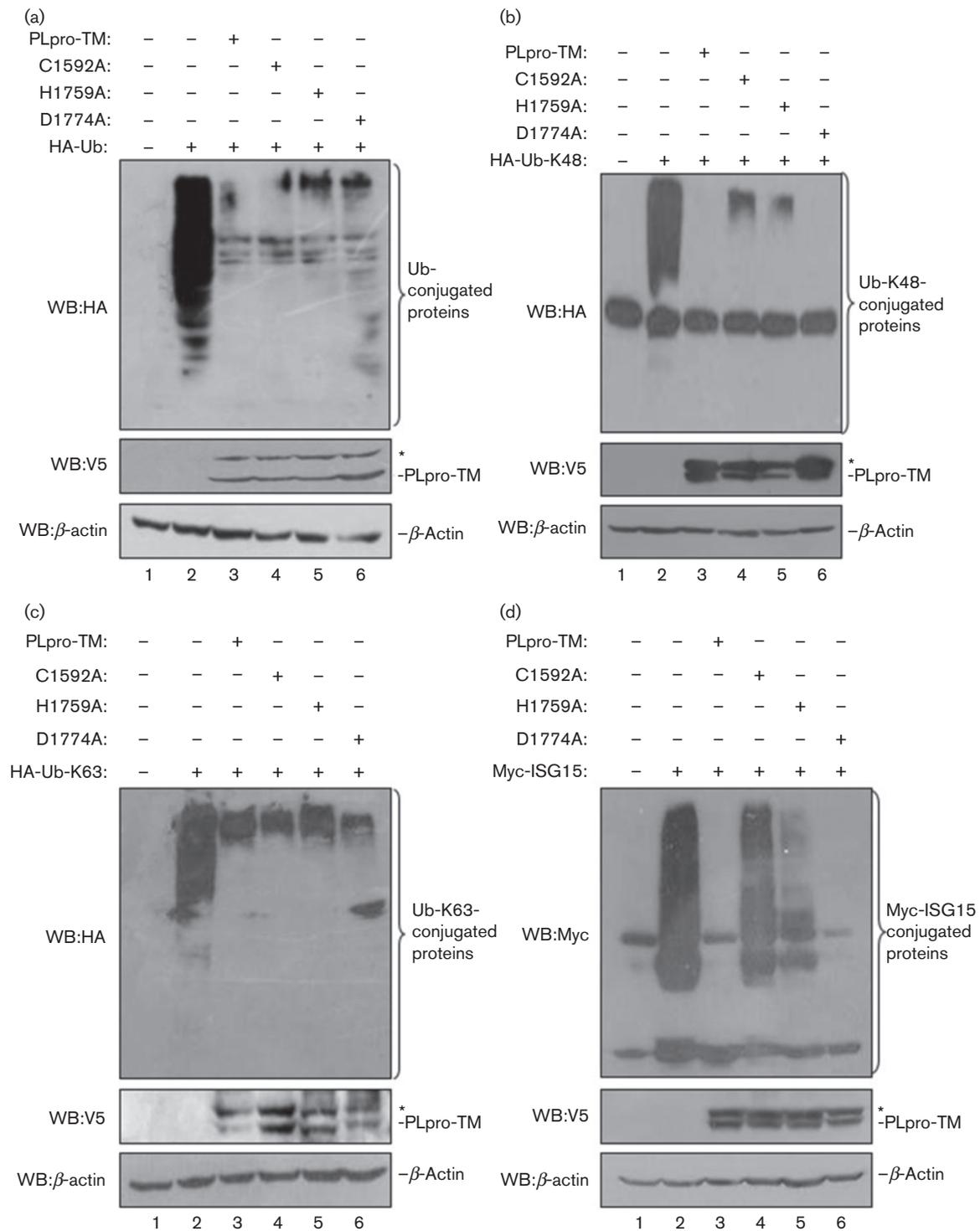


Fig. 5. MERS-CoV PLpro-TM has DUB activity. (a) HEK293T cells were transfected with HA-tagged Ub and either MERS-CoV PLpro-TM or its catalytic mutants (C1592A, H1759A and D1774A). Cell lysates were subjected to Western blotting using anti-HA antibody to visualize HA-tagged Ub-conjugated proteins (top panel) and anti-V5 antibody (middle panels) to visualize the expression of the PLpro-TM constructs. β -Actin was detected by Western blotting as protein loading control (bottom panel). (b, c) HA-Ub-K48 (b) or HA-Ub-K63 (c) was transfected with MERS-CoV PLpro-TM or its catalytic mutants into HEK293T cells. HA-tagged Ub-K48- and HA-tagged Ub-K63-conjugated proteins were assayed as described above. (d) HEK293T cells were cotransfected with Myc-ISG15, Ube1L, UbcH8 and PLpro-TM or its catalytic mutants and treated with MG132 6 h later. The ISG15-conjugated proteins were assayed as described above. The asterisk indicates the putative glycosylated form of PLpro-TM.

PLpro exhibited DUB activity on ubiquitinated proteins without any specificity for either K48 or K63 linkage.

It has been reported that both SARS-CoV PLpro and NL63 PLP2 have deISGylation activities (Clementz *et al.*, 2010; Lindner *et al.*, 2007; Sun *et al.*, 2010). To examine whether MERS-CoV PLpro could influence the ISGylation of cellular proteins, a combination of Ube1L, UbcH8 and Myc-ISG15 together with either WT PLpro-TM or the catalytic mutants was transfected into HEK293T cells. The cell lysates were subjected to Western blotting using anti-Myc antibody to detect the ISGylation of the cellular proteins. The ISGylation of substrate proteins was markedly decreased by MERS-CoV PLpro-TM (Fig. 5d, lane 3), indicating that MERS-CoV PLpro acted as a deISGylation enzyme. Interestingly, introducing mutations into the catalytic site D1774 did not have any effect on deISGylation enzyme activity (Fig. 5d, lane 6), indicating that D1774 is not required for deISGylation, which is similar to DUB activity against Ub (Fig. 5a, lane 6). However, in contrast to PLpro DUB, the catalytic mutants C1592A and H1759A of PLpro lost almost all deISGylation activity (Fig. 5d, lanes 4 and 5), indicating that the catalytic sites C1592 and H1759 are required for deISGylation. These results definitely indicated that MERS-CoV PLpro had potent DUB activity of both Ub and ISG15-conjugated proteins in host cells, which may be associated with its protease activity.

MERS-CoV PLpro is an IFN antagonist

We previously reported that PLpros of human CoVs, SARS-CoV and NL63-CoV, acted as IFN antagonists (Clementz *et al.*, 2010; Devaraj *et al.*, 2007; Sun *et al.*, 2010, 2012). To determine if MERS-CoV PLpro was an IFN antagonist, which may contribute to inhibiting the expression of type I IFN, HEK293T cells were transfected with the plasmids expressing WT PLpro-TM or its catalytic mutants separately, along with an IFN- β promoter-driven luciferase reporter and Flag-RIG-IN (N-terminal helicase domain of RIG-I as its constitutively active mutant) that was used to activate the IFN- β promoter activity. As shown in Fig. 6a, the IFN- β promoter was activated 100-fold when the cells were transfected with RIG-IN, whereas the IFN- β promoter activated by RIG-IN was completely inhibited in the presence of MERS-CoV PLpro-TM, which was similar to the previously reported IFN antagonist activity of SARS-CoV PLpro and NL63-CoV PLP2 (Clementz *et al.*, 2010; Devaraj *et al.*, 2007; Sun *et al.*, 2010). To determine if MERS-CoV PLpro catalytic activity is essential for inhibition of IFN expression, the catalytic mutants of MERS-CoV PLpro-TM (C1592A, H1759A and D1774A) were transfected together with the IFN- β -Luc and RIG-IN plasmids into HEK293T cells; we found that all catalytic mutants of PLpro-TM had inhibition effects on RIG-IN-activated IFN- β promoter-driven luciferase activity (Fig. 6a). These data demonstrated that MERS-CoV PLpro-TM was an IFN antagonist, which was independent of its protease activity.

Cellular transcription factors, NF- κ B, IRF3 and AP-1 play critical roles in the transcriptional control of the IFN- β promoter (Barber, 2011; Dev *et al.*, 2011; Marsili *et al.*, 2012). To determine whether MERS-CoV PLpro inhibition of the activation of the IFN- β promoter was related to its suppressive effect on NF- κ B, IRF3 or AP-1 activation, we performed a luciferase reporter assay to analyse the transcriptional activity of NF- κ B, IRF3 and AP-1 in the presence of MERS-CoV PLpro. As shown in Fig. 6(b, c), we found that the activation of IRF3 and NF- κ B was significantly inhibited by WT MERS-CoV PLpro-TM. However, the AP-1-dependent IFN- β expression was comparable to the control vector when it was cotransfected with MERS-CoV PLpro-TM (Fig. 6d). These findings suggested that MERS-CoV PLpro-TM suppressed IFN- β transcription by interfering with the IRF3- and NF- κ B-mediated IFN expression signalling pathway.

To determine how MERS-CoV PLpro-TM inhibited the activation of IFN production signalling, Western blotting and immunofluorescence assays were conducted to detect the phosphorylation status and subcellular localization of IRF3 in PLpro-TM-transfected cells. We found that MERS-CoV PLpro-TM significantly inhibited the phosphorylation of IRF3, which was activated by RIG-IN (Fig. 6e, lane 4). Compared with the control, it had no inhibitory effect on the RIG-IN-mediated phosphorylation of IRF3, which was mock-transfected with PLpro-TM (Fig. 6e, lane 2). Interestingly, consistent with the inhibition on IRF3 phosphorylation, we observed that RIG-IN-induced IRF3 nuclear translocation (Fig. 6f) was markedly blocked in PLpro-TM-expressing cells. In contrast, IRF3 was exclusively located in the nuclei of the cells when the cells were transfected with RIG-IN only. Taken together, the data suggested that MERS-CoV PLpro inhibited IFN- β transcription and expression through blocking the phosphorylation and nuclear translocation of IRF3.

DISCUSSION

It is noteworthy that there were six human CoVs identified before 2012, but of these only SARS-CoV causes severe pulmonary disease with a high mortality rate in humans. In June 2012, MERS-CoV was isolated from a 60-year-old Saudi Arabian patient who died from acute respiratory distress syndrome with multiple organ failure (Zaki *et al.*, 2012). Person-to-person transmission of MERS-CoV could spread the disease globally and become a serious health issue (Assiri *et al.*, 2013). Currently, there are no specific anti-viral drugs to prevent the transmission of MERS-CoV or provide a cure for the disease.

In this study, we firstly identified the proteolytic processing activity of PLpro of MERS-CoV, the protease that can recognize and process the cleavage sites of nsp1-2, nsp2-3 and nsp3-4, and the catalytic residues required for MERS-CoV PLpro. Secondly, the LXGG consensus cleavage sites at the N terminus of pp1a/1ab, which is generally

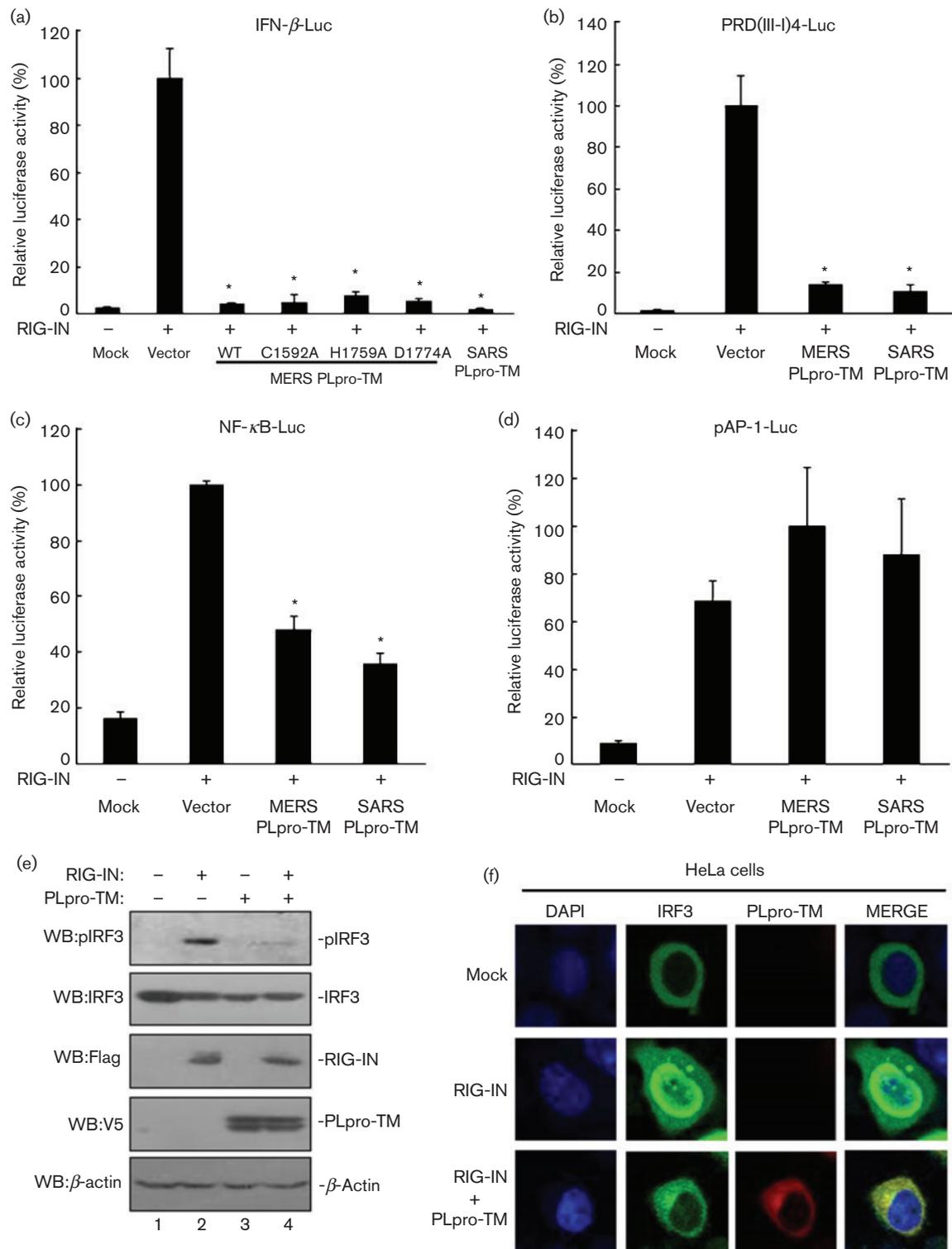


Fig. 6. MERS-CoV PLpro-TM inhibits IFN- β expression. (a) MERS PLpro-TM or catalytic mutants (C1592A, H1759A and D1774A) were transfected together with IFN- β -Luc, pRL-TK and RIG-IN (N-terminal helicase domain of RIG-I as its constitutively active mutant) into HEK293T cells. SARS-CoV PLpro-TM acted as a positive control. The cells were harvested and subjected to a dual-luciferase assay as described in Methods. (b, c and d) HEK293T cells were cotransfected with MERS PLpro-TM, together with RIG-IN, pRL-TK and PRD(III-I)4-Luc (b), pNF- κ B-Luc (c) or pAP-1-Luc (d), followed by the assay performed in (a). (e) RIG-IN and PLpro-TM were cotransfected into HEK293T cells. The cells were lysed and subjected to Western blotting using anti-pIRF3 antibody to visualize phosphorylated IRF3, anti-IRF3 antibody to visualize total IRF3, anti-Flag

Fig. 6. (cont.) antibody to visualize RIG-IN, and anti-V5 antibody to visualize PLpro-TM expression. β -Actin was detected by Western blotting as a protein loading control. (f) MERS-CoV PLpro-TM inhibits IRF3 nuclear translocation. HeLa cells were transfected or mock-transfected with RIG-IN and MERS-CoV PLpro-TM. Twenty-four hours later, an immunofluorescence assay was performed using a confocal microscope. Asterisks indicate statistical significance ($P < 0.05$).

essential for MERS-CoV PLpro-mediated processing, were characterized. We also demonstrated that MERS-CoV PLpro has DUB enzyme activity as observed in other human and animal CoVs (Barretto *et al.*, 2005; Chen *et al.*, 2007; Clementz *et al.*, 2010; Frieman *et al.*, 2009; Harcourt *et al.*, 2004; Sun *et al.*, 2010, 2012; Ziebuhr *et al.*, 2007). Similar to human SARS-CoV PLpro and NL63-CoV PLP2, MERS-CoV PLpro is a viral deubiquitinating enzyme, which acts on both K48- and K63-linked ubiquitination and ISG15-linked ISGylation. Finally, we confirmed that MERS-CoV PLpro acts as an IFN antagonist through blocking the phosphorylation and nuclear translocation of IRF3. The characterization of protease, DUB and IFN antagonist activities of MERS-CoV PLpro would reveal the multilayered interactions between virus and host, and might contribute to the development of new strategies targeting PLpro for an effective prevention of MERS-CoV infection.

One of the intriguing findings in this study is that MERS-CoV PLpro is sufficient to process the substrates. By comparing the sequences with other CoVs, we found the cleavage site sequences at the N terminus of pp1a/1ab are highly conserved. MERS-CoV PLpro processed the polyproteins nsp1-2, nsp2-3 and nsp3-4 at the consensus cleavage site LXGG, which is also the target sequence recognized by many cellular DUB enzymes (Barretto *et al.*, 2005; Sulea *et al.*, 2005). Our results demonstrate that LXGG is an important determinant element for PLpro recognition and processing, which is in agreement with previous studies (Barretto *et al.*, 2005; Chen *et al.*, 2007). To our surprise, the consensus cleavage site LKGG in nsp2-3 is not absolutely required for PLpro recognition and processing, suggesting that other residues around the site would also be involved in PLpro recognition and processing. More studies with more mutations around the consensus cleavage site are warranted. Proteolytic processing of the replicase polyprotein, which is essential for the proper assembly of the replication complex, plays a critical role in mediating RNA synthesis (Gosert *et al.*, 2002; Kim *et al.*, 1995; Sawicki *et al.*, 2005). A previous study reported that MHV-CoV PLP1-mediated processing at CS1 and CS2 was important for efficient viral replication (Kanjana-haluthai *et al.*, 2003). Based on the findings of this study, our future research will aim to explore whether PLpro activity and processing of the substrates are necessary for MERS-CoV replication.

Another interesting result from this study is the demonstration of MERS-CoV PLpro DUB activity, which is independent of the catalytic triad. Our results revealed that MERS-CoV PLpro is a new DUB enzyme that is encoded

by the viral genome and may play important roles in CoV replication and pathogenesis. Cellular DUBs alter the fate of proteins in specific signalling pathways of the cell (Katz *et al.*, 2010; Reyes-Turcu *et al.*, 2009), for example deubiquitination may protect the ubiquitinated proteins from degradation, and affect the proteins which are modified by Ub-like proteins, such as ISGs (van der Veen & Ploegh, 2012). For instance, a central gatekeeper in inflammation and immunity, A20, has DUB activity and removes K63-linked polyubiquitin lines from RIP1, TRAF6, RIP2 and NEMO, which results in the negative regulation of the innate immune response (Coornaert *et al.*, 2009; Ma & Malynn, 2012). Cellular proteins, DUBA and CYLD, also negatively regulate the innate immune responses (Kayagaki *et al.*, 2007; Sun, 2008, 2010). Furthermore, a lot of viral DUBs have similar structures to known cellular DUBs, such as USP14 and HAUSP, which have been identified as negative regulators of innate immunity (Chen *et al.*, 2010; Kim *et al.*, 2009; Ratia *et al.*, 2006; Wang *et al.*, 2011). For example, human CoV NL63, SARS-CoV and MERS-CoV, reported in this study, have contributed to encoding DUBs, and likely modulating the innate immune responses. We previously reported that human CoV NL63 PLP2 reduced the ubiquitinated forms of STING, RIG-I, TBK1 and IRF3 (Sun *et al.*, 2012). However, the underlying mechanisms by which MERS-CoV PLpro affects viral replication and regulation of host innate immunity remain largely unknown. Here, for the first time, we report that MERS-CoV PLpro inhibits the conjugation of Ub, which is independent of the catalytic triad (C1592–H1759–D1774), and PLpro may target the Ub-proteasome pathway to promote virus replication and block the innate immunity. There is another possibility that PLpro may affect the proteins that are post-translationally modified by the Ub-like modifier, such as ISG15. We found MERS-CoV PLpro has deISGylation activity as a large number of ISG15-conjugated proteins are inhibited when PLpro is co-expressed with ISG15. These results suggested that MERS-CoV PLpro may affect protein subcellular localization, protein activity and stability as well as signal transduction through these modifications and plays a critical role in the innate immune responses.

MERS-CoV causes a highly lethal infectious disease in human that is characterized by an aberrant immune response. The production of type I IFN, the major component of antiviral innate immunity, is firstly inhibited by MERS-CoV infection (Zielecki *et al.*, 2013). Furthermore, MERS-CoV fails to elicit strong type I or III IFN or pro-inflammatory innate immune responses (Chan *et al.*, 2013; Kindler *et al.*, 2013). IRF3 and NF- κ B are the key

transcription factors for IFN- β expression (Barber, 2011; Dev *et al.*, 2011; Marsili *et al.*, 2012). The results of this study indicated that MERS-CoV PLpro markedly reduces the activation of the IFN- β , IRF3 and NF- κ B promoters, which are induced by RIG-IN, indicating that MERS-CoV PLpro acts as an IFN antagonist. The immunofluorescence analysis shown in Fig. 6(f) indicates that IRF3 nuclear translocation is disrupted and remains located in the cytoplasm following cotransfection with PLpro and RIG-IN. This is consistent with previous studies of other CoVs (Devaraj *et al.*, 2007; Xing *et al.*, 2013). In line with this, Western blotting indicates that the phosphorylation of IRF3 is also blocked by PLpro. Thus, MERS-CoV may possess the ability to dampen human innate immune responses by PLpro through blocking the activation of IRF3 and regulation of the IFN response. While the underlying mechanisms currently remain unclear, further experiments are needed to explore the detailed mechanisms of how MERS-CoV PLpro regulates host antiviral innate immune responses.

In summary, the characterization of proteolytic processing, DUB and IFN antagonist activities of MERS-CoV PLpro would reveal the multilayered interactions between MERS-CoV and its host. These findings might be applicable for developing new strategies targeting PLpro for the effective control of MERS-CoV infection and investigating the roles of a viral DUB activity in CoV replication and pathogenesis.

METHODS

Cell and plasmid DNA. HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) FCS supplemented with penicillin (100 U ml⁻¹) and streptomycin (100 μ g ml⁻¹). The reporter plasmids IFN- β -Luc, PRD (III-I)4-Luc, NF- κ B-Luc and pAP-1-Luc, constructs HA-tagged Ub, HA-tagged Ub-K63 and HA-tagged Ub-K48 (all other K residues were mutated), and plasmids Ube1L, UbcH8, Myc-ISG15 and Flag-RIG-IN were described previously (Clementz *et al.*, 2010; Devaraj *et al.*, 2007; Sun *et al.*, 2012).

Generating MERS-CoV PLpro-TM, substrate constructs and site-directed mutants. MERS-CoV PLpro-TM (Fig. 1a; amino acids 1474–2359 of pp1a of MERS-CoV EMC/2012 strain; GenBank accession number JX869059.1) and its three substrate constructs nsp1-2 (amino acids 1–330 of pp1a), nsp2-3 (amino acids 676–1005 of pp1a) and nsp3-4 (amino acids 2581–2908 of pp1a) described in Fig. 1(b) were generated in this study. To obtain high-level expression in eukaryotic cells, we optimized the codon usage of these constructs based on the human codon usage frequency and the potential splicing sites and removed polyadenylation signal sequences from the coding regions. The optimized DNA was synthesized and cloned into pcDNA3.1-V5/HisB (Invitrogen) between the *Bam*HI and *Eco*RI sites with in-frame fusion with a C-terminus V5 tag for PLpro-TM (Fig. 1a) or in-frame fusion with a Flag tag at the N terminus and a V5 tag at the C terminus for substrate constructs (Fig. 1b). To generate the catalytically inactive mutants of MERS-CoV PLpro-TM (C1592A, H1759A and D1774A) in Fig. 1(a) or specific mutants at the cleavage sites of substrates (Fig. 4a), we designed mutagenic primers (sequences are available on request) according to the methods previously reported (Zheng

et al., 2004) and incorporated them into newly synthesized DNA using the QuikChange site-directed mutagenesis protocol (Stratagene). All introduced mutations were confirmed by DNA sequencing.

Cell-based trans-cleavage assay of MERS-CoV PLpro-TM.

PLpro-TM or the catalytic mutants (4.5 μ g) and the indicated substrates (0.5 μ g) were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours post-transfection, the cells were lysed for Western blotting to detect the cleavage products, as previously described (Sun *et al.*, 2012).

Luciferase detection assay. The reporter plasmids (IFN- β -Luc, PRD(III-I)4-Luc, NF- κ B-Luc or pAP-1-Luc) and Flag-RIG-IN, were cotransfected together with MERS-CoV PLpro-TM or the corresponding catalytic mutants into HEK293T cells using Lipofectamine 2000 according to the manufacturer's protocol. Luciferase assay was performed as described previously (Sun *et al.*, 2012; Xing *et al.*, 2013). SARS-CoV PLpro-TM was used as the positive control of inhibitor for IFN- β expression (Clementz *et al.*, 2010). Data between Vector and PLpro were subjected to unpaired, two-tailed Student's *t* test using the Microsoft SPSS 12.0 software. A *P* value of <0.05 was considered statistically significant (Kiefer *et al.*, 2009; Vaux *et al.*, 2012).

Assay of DUB and deISGylation activities. The assay of MERS-CoV PLpro-TM DUB was performed as described previously (Clementz *et al.*, 2010; Xing *et al.*, 2013). The effect of MERS-CoV PLpro-TM on ISG15-conjugated proteins in cultured cells was assessed as previously described (Evans *et al.*, 2004; Zhao *et al.*, 2005).

IRF3 phosphorylation detection. To assess IRF3 phosphorylation, we transfected Flag-RIG-IN (1.2 μ g) in the presence or absence of V5-tagged PLpro-TM (3 μ g) into HEK293T cells. IRF3 phosphorylation detection assay was performed as described previously (Xing *et al.*, 2013).

Immunofluorescence assay. HeLa cells were grown to confluence in a six-well plate. Plasmid DNA expressing Flag-RIG-IN (1.2 μ g per well) was transfected in the presence or absence of MERS-CoV PLpro-TM. Immunofluorescence assay was performed as described previously (Xing *et al.*, 2013).

Sequence comparison and structure analysis of MERS-CoV

PLpro. The protocol for sequence comparison and structure analysis of MERS-CoV PLpro was previously described (Barretto *et al.*, 2005; Ratia *et al.*, 2006). Briefly, the amino acid sequences of the PLpros from representative CoVs (Fig. 2a) were aligned with the MERS-CoV PLpro catalytic core domain (amino acid sequences 1476 to 1804) as the reference sequence using the program CLUSTAL 2.1. The 3D structure of MERS-CoV PLpro domains was modelled based on the structure of human SARS-CoV PLpro (PDB code, 2FE8) (Ratia *et al.*, 2006) using SWISS-MODEL (Kiefer *et al.*, 2009), an automated protein structure homology-modelling server (<http://beta.swissmodel.expasy.org/worksapce>).

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