Catalytic Function and Substrate Specificity of the Papain-Like Protease Domain of nsp3 from the Middle East Respiratory Syndrome Coronavirus

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ABSTRACT

The papain-like protease (PLpro) domain from the deadly Middle East respiratory syndrome coronavirus (MERS-CoV) was overexpressed and purified. MERS-CoV PLpro constructs with and without the putative ubiquitin-like (UBL) domain at the N terminus were found to possess protease, deubiquitinating, deISGylating, and interferon antagonism activities in transfected HEK293T cells. The quaternary structure and substrate preferences of MERS-CoV PLpro were determined and compared to those of severe acute respiratory syndrome coronavirus (SARS-CoV) PLpro, revealing prominent differences between these closely related enzymes. Steady-state kinetic analyses of purified MERS-CoV and SARS-CoV PLpros uncovered significant differences in their rates of hydrolysis of 5-aminomethyl coumarin (AMC) from C-terminally labeled peptide, ubiquitin, and ISG15 substrates, as well as in their rates of isopeptide bond cleavage of K48- and K63-linked polyubiquitin chains. MERS-CoV PLpro was found to have 8-fold and 3,500-fold higher catalytic efficiencies for hydrolysis of ISG15-AMC than for hydrolysis of the Ub-AMC and Z-RLRGG-AMC substrates, respectively. A similar trend was observed for SARS-CoV PLpro, although it was much more efficient than MERS-CoV PLpro toward ISG15-AMC and peptide-AMC substrates. MERS-CoV PLpro was found to process K48- and K63-linked polyubiquitin chains at similar rates and with similar debranching patterns, producing monoubiquitin species. However, SARS-CoV PLpro much preferred K48-linked polyubiquitin chains to K63-linked chains, and it rapidly produced di-ubiquitin molecules from K48-linked chains. Finally, potent inhibitors of SARS-CoV PLpro were found to have no effect on MERS-CoV PLpro. A homology model of the MERS-CoV PLpro structure was generated and compared to the X-ray structure of SARS-CoV PLpro to provide plausible explanations for differences in substrate and inhibitor recognition.

IMPORTANCE

Unlocking the secrets of how coronavirus (CoV) papain-like proteases (PLpros) perform their multifunctional roles during viral replication entails a complete mechanistic understanding of their substrate recognition and enzymatic activities. We show that the PLpro domains from the MERS and SARS coronaviruses can recognize and process the same substrates, but with different catalytic efficiencies. The differences in substrate recognition between these closely related PLpros suggest that neither enzyme can be used as a generalized model to explain the kinetic behavior of all CoV PLpros. As a consequence, decoding the mechanisms of PLpro-mediated antagonism of the host innate immune response and the development of anti-CoV PLpro enzyme inhibitors will be a challenging undertaking. The results from this study provide valuable information for understanding how MERS-CoV PLpro-mediated antagonism of the host innate immune response is orchestrated, as well as insight into the design of inhibitors against MERS-CoV PLpro.

Coronaviruses (CoVs) can infect and cause diseases in a wide range of vertebrates, including humans and a variety of livestock, poultry, and domestic animals. Diseases caused by coronaviruses range from respiratory to enteric, hepatic, and neurological, and they have variable incidences and clinical severities (1, 2). Until 2012, five human coronaviruses (HCoVs) were known. The first two human coronaviruses, HCoV-229E and HCoV-OC43, were discovered in the mid-1960s as the causative agents of mild respiratory infections (3, 4). In 2003, a new human coronavirus was identified as the causative agent of the first global pandemic of the new millennium. This new human coronavirus was named severe acute respiratory syndrome coronavirus (SARS-CoV), as it caused a pathogenic respiratory infection in over 8,000 humans in nearly 30 countries and exhibited a case-fatality rate of nearly 10% (5–8). This event prompted interest in coronavirus research, resulting in the discovery of two additional human coronaviruses (HCoV-NL63, in 2004 [9], and HCoV-HKU1, in 2005 [10]).

However, because of the lack of effective diagnostic methods, it was not until recently that human coronaviruses, with the exception of SARS-CoV, were found to be circulating in the human population, and they are now implicated in contributing a significant percentage of known human respiratory tract infections (11). Most recently, nearly 10 years after the discovery of SARS-CoV, a new human coronavirus was discovered in the Middle East, and thus far it has a significantly higher case-fatality rate

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(~30%) than that of SARS-CoV (12, 13). The new human coronavirus was named Middle East respiratory syndrome coronavirus (MERS-CoV) (formerly HCoV-EMC/2012, for Erasmus Medical Center) and is associated with severe acute respiratory infection (SARI), often combined with kidney failure (14). So far, there have been 837 laboratory-confirmed cases of MERS-CoV infection in 20 countries, with the first case in the United States, in Indiana, reported in 2 May 2014 (15). The resemblance of the MERS-CoV situation to the initial stages of the SARS-CoV pandemic has raised important public health concerns and research interest (16). As a result, the complete genome sequence has been obtained, animal models are being developed, and phylogenetic, evolutionary, receptor interaction, and tissue tropism analyses are now becoming available (14, 17–19).

As with all coronaviruses, MERS-CoV is an enveloped, positive-sense RNA virus with a genome of nearly 30 kb (14). Similar to SARS-CoV, MERS-CoV belongs to the genus *Betacoronavirus*, but it constitutes a sister species in group C (14). The complete genomic analysis suggests that MERS-CoV is phylogenetically related to bat coronaviruses HKU4 and HKU5, previously found in lesser bamboo bats and Japanese pipistrelle bats from Hong Kong, respectively (14, 16). As observed previously with the zoonotic acquisition of HCoV-OC43 and SARS-CoV, the close genomic relationship of MERS-CoV PLpro to bat coronaviruses HKU4 and HKU5 suggests a zoonotic origin from bat coronaviruses (17). Recently, a number of animals, including dromedary camels and Egyptian cave bats, have been considered potential intermediate host animals for the animal-to-human transmission of MERS-CoV; however, more research is necessary for confirmation (18–21). Alarmingly, human-to-human transmission has now been reported, with a higher prevalence in immunocompromised patients or patients with underlying diseases (22–24).

The host immune response to viral infection has been linked directly to MERS-CoV outcomes in patients (25). As a mechanism to promote viral replication, coronaviruses encode proteins that can actively antagonize cellular signaling pathways associated with the host establishment of an antiviral state (26). The coronavirus nsp3 multifunctional protein contains numerous domains, including the interferon (IFN) antagonist papain-like protease (PLpro) domain. PLpro is a multifunctional cysteine protease that hydrolyzes peptide and isopeptide bonds in viral and cellular substrates, an essential function for coronavirus replication. In SARS-CoV, the main roles of PLpro enzymatic activity involve processing of the replicase polyprotein at the N terminus of pp1a, releasing the nonstructural proteins nsp1, nsp2, and nsp3 (27). Importantly, because of the essentiality of these events, inhibition of PLpro enzymatic activity is an ongoing approach for the development of anticoronaviral drugs (28–38). Other enzymatic activities involve the removal of the cellular substrates ubiquitin (Ub), termed deubiquitination (DUB), and interferon-stimulated gene 15 (ISG15), termed deISGylation, from host cell proteins (reviewed in reference 39). Processing of the replicase polyprotein (40, 41) and cellular DUB and deISGylation activities (41, 42) were also recently characterized for the PLpro domain from MERS-CoV. The DUB and deISGylating activities of PLpro have important implications during the PLpro-mediated IFN antagonism of the host innate immune response. We recently demonstrated that the PLpro domain from MERS-CoV exhibits both DUB and deISGylating activities in host cells and that these activities block the production of IFN- β in transfected cells (42). Sim-

ilarly, Yang et al. showed that MERS-CoV PLpro blocks the signaling pathway that leads to the activation of IFN regulatory factor 3 (IRF3) (41).

Most of the findings involving the cellular functions of PLpro were initially elucidated with the PLpro domain from SARS-CoV, and later with those of HCoV-NL63 and mouse hepatitis virus (MHV) (43–48). However, the exact mechanism by which coronavirus PLpros perform their multifunctional roles via the recognition and catalysis of viral and cellular substrates remains elusive. The relatively low amino acid conservation among HCoV PLpro domains suggests that there are mechanistic aspects unique to each enzyme. Therefore, in order to better understand the mechanism behind CoV PLpro-mediated antagonism of the innate immune response and to develop anticoronaviral inhibitors, further research must emphasize the enzymatic characterization of the PLpro domains from newly discovered human coronaviruses. Here we report the purification, biochemical and kinetic characterization, and substrate specificity of the PLpro domain from MERS-CoV nsp3. Detailed comparisons between MERS-CoV PLpro and SARS-CoV PLpro steady-state kinetic parameters, substrate preferences, and inhibition are also presented and shed light on the convergent and divergent functional roles of these two enzymes.

MATERIALS AND METHODS

Expression and enzymatic activity of MERS-CoV PLpro N-terminal deletion constructs in HEK293T cells. (i) **Cells and transfections.** HEK293T cells and BHK-21 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) and 2% glutamine. Transfections were performed with 70% confluent cells in cell culture plates (Corning) for BHK-21 cells, using Lipofectamine 2000, and in cell bind plates (Corning) for HEK293T cells, using TransIT-LT1 reagent (Mirus) according to the manufacturer's protocols.

(ii) **Expression constructs.** The MERS-CoV PLpro (pcDNA-MERS-PLpro) expression plasmid and generation of the catalytic mutant were described previously (40). Twenty-, 40-, and 60-amino-acid (aa) N-terminal deletions of the MERS-CoV PLpro ubiquitin-like domain (UBL) (designated N20, N40, and N60, respectively) with an in-frame C-terminal V5 tag were generated by PCR amplification from pcDNA-MERS-PLpro, using a forward primer (N20-Fwd, AGTGAATTCACCATGAAA AATACTTATCGGTCTC; N40-Fwd, AGTGAATTCACCATGGATACT ATTCCCGACGAG; or N60-Fwd, AGTGAATTCACCATGGATGAGAC TAAGGCCCTG) and the reverse primer PLpro-Rev (CGGGTTAACT CATGTTGAATCCAATC), and then ligated into the pcDNA3.1-V5/His-B vector (Invitrogen). For *trans*-cleavage assay, the nsp2/3-GFP substrate construct was kindly provided by Ralph Baric (University of North Carolina) (44). For luciferase assay experiments, we used IFN- β -Luc, provided by John Hiscott (Jewish General Hospital, Montreal, Canada), and the *Renilla* luciferase expression plasmid pRL-TK (Promega), as previously described (45). The pEF-BOS MDA5 (Addgene) expression plasmid was a gift from Kate Fitzgerald (University of Massachusetts Medical School). The epitope-tagged constructs for the DUB and deISGylation assays, including pcDNA3.1-Flag-Ub (provided by Adriano Marchese, Loyola University Medical Center), pcDNA3-myc6-mISG15 (a gift from Min-Jung Kim, Pohang University of Science and Technology, Pohang, Republic of Korea), and pcDNA3-Ube1L, pcDNA3-UbcH8, and pcDNA-Herc5, expressing the E1, E2, and E3 ISG15-conjugating enzymes, respectively (provided by Robert M. Krug, University of Texas), were used as described below.

DeISGylation and DUB activity assays. For deISGylation assay, BHK-21 cells in 24-well plates were cotransfected with 200 ng of MERS-CoV PLpro plasmid, 250 ng pISG15-myc, 125 ng pUbcH8, 125 ng pUbe1L, and 125 ng pHerc5. For DUB assay, HEK293T cells were trans-

fectected with 300 ng Flag-Ub plasmid and 1 μ g MERS-CoV PLpro plasmid. At 18 h posttransfection, cells were lysed with lysis buffer A (4% SDS, 3% dithiothreitol [DTT], and 65 mM Tris, pH 6.8). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Following transfer, the membrane was blocked using 5% dried skim milk in TBST buffer (0.9% NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween 20) for 2 h at ambient temperature. For deISGylation assay, the membrane was incubated with mouse anti-myc antibody (MBL) at a dilution of 1:2,500 overnight at 4°C. For DUB assay, the membrane was incubated with mouse anti-Flag M2 antibody (Sigma) at a dilution of 1:2,000 for 1 h at ambient temperature. The membrane was washed 3 times for 10 min in TBST buffer. The membrane was then incubated with secondary goat anti-mouse-horseradish peroxidase (HRP) antibody at a dilution of 1:10,000 (Amersham) for 1 h at ambient temperature. After washing in TBST buffer, detection was performed using Western Lighting chemiluminescence reagent Plus (PerkinElmer) and visualized using the ProteinSimple FluorChem E system. The membrane was probed with mouse anti-V5 antibody (Invitrogen) at a dilution of 1:5,000 to verify the expression of PLpro.

Luciferase assay. HEK293T cells in 24-well plates were transfected with 50 ng *Renilla* luciferase, 100 ng IFN- β -luc, and increasing doses of MERS-CoV PLpro UBL-deleted mutants (25, 50, and 100 ng) or 100 ng wild-type (WT) or catalytic mutant MERS-CoV PLpro expression plasmid. For stimulation, the cells were transfected with 150 ng pEF-BOS MDA5. At 16 h posttransfection, cells were lysed using 1 $\times$ passive lysis buffer (Promega). The firefly and *Renilla* luciferase activities were measured using a dual-luciferase reporter assay system (Promega) and a luminometer (Veritas). Results were normalized to the *Renilla* luciferase expression control. Experiments were performed in triplicate. Remaining lysates were incubated with lysis buffer and analyzed by SDS-PAGE for the detection of PLpro expression as described above.

Construction of MERS-CoV PLpro expression plasmid. The PLpro catalytic domain of nsp3 (aa 1484 to 1802) from MERS-CoV was synthesized by Bio Basic Inc., with codon optimization for *Escherichia coli* expression. The gene was inserted into Bio Basic's standard vector pUC57. The MERS-CoV PLpro coding region (aa 1484 to 1802) was amplified using forward and reverse primers containing complementary sequences to an expression plasmid, pEV-L8, and PLpro at the 5' and 3' ends, respectively. The PCR amplicon was then inserted into the pEV-L8 vector by ligation-independent recombinant cloning, using linearized pEV-L8 vector digested by SspI and XL1-Blue supercompetent cells. The resulting MERS-CoV pEV-L8-PLpro expression plasmid was transformed into *E. coli* BL21(DE3) for protein expression.

Expression and purification of MERS-CoV PLpro. Four liters of *E. coli* BL21(DE3) cells containing MERS-CoV pEV-L8-PLpro (aa 1484 to 1802) was grown for 24 h at 25°C in medium containing 3 g KH₂PO₄, 6 g Na₂HPO₄, 20 g tryptone, 5 g yeast extract, and 5 g NaCl, pH 7.2, supplemented with 0.2% lactose, 0.6% glycerol, 0.05% glucose, and 50 μ g/ml kanamycin. Approximately 29 g of cells was harvested by centrifugation (18,500 $\times$ g for 30 min at 4°C) and resuspended in 125 ml of buffer A (20 mM Tris, pH 7.5, 500 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol [BME], and 10% glycerol) containing lysozyme and DNase I. The resuspended cells were lysed on ice via sonication, and the cell debris was pelleted by centrifugation. The clarified lysate was loaded at 2 ml/min onto a 5-ml HisTrap FF column (GE Healthcare) precharged with Ni²⁺. Unbound proteins were washed with 5 column volumes (CV) of buffer A. Bound proteins were eluted using a linear gradient of 0% to 100% buffer B (20 mM Tris, pH 7.5, 500 mM NaCl, 500 mM imidazole, 10 mM BME, and 10% glycerol) at 2 ml/min, followed by a wash with 5 CV of 100% buffer B. Fractions containing MERS-CoV PLpro were concentrated, buffer exchanged into buffer C (20 mM Tris, pH 7.5, 10 mM BME, and 10% glycerol), and loaded onto a Mono Q 10/100 GL (GE Healthcare) column equilibrated with buffer C. MERS-CoV PLpro was eluted with a linear gradient of 0% to 60% buffer D (20 mM Tris, pH 7.5, 500 mM NaCl, 10 mM BME, and 10% glycerol). Fractions containing MERS-CoV PLpro

were concentrated to 500 μ l and loaded onto a HiLoad 26/60 Superdex 75 column (GE Healthcare) equilibrated with final buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM DTT, and 5% glycerol). For enzyme kinetic studies, the His₆ tag was removed via tobacco etch virus (TEV) protease cleavage prior to the MonoQ chromatography step. Aliquots of 100 μ l at 10 mg/ml were flash-frozen in liquid nitrogen in buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM DTT, and 20% glycerol.

Expression and purification of SARS-CoV PLpro. The PLpro catalytic domain of nsp3 from SARS-CoV was expressed and purified according to our previously published methods (28).

SEC-MALS analysis. Aliquots of 100 μ l of MERS-CoV PLpro at concentrations of 4.2 mg/ml, 2.1 mg/ml, and 1.0 mg/ml in buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM TCEP, and 5% glycerol) were used for analytical size-exclusion chromatography (SEC) coupled with multiangle light scattering (MALS) analyses. SEC was performed using a GE Healthcare Superdex 75 analytical gel filtration column at a flow rate of 0.5 ml/min and was coupled to a Dawn Heleos MALS instrument (Wyatt Technology) and an Optilab rEX instrument (Wyatt Technology). The online measurement of the intensity of Rayleigh scattering as a function of the angle as well as the differential refractive index of the eluting peak in SEC was used to determine the weight-average molecular weight ($\bar{M}_w$) of eluted oligomers and protein complexes, using Astra (Wyatt Technology) software. The number-average molecular weight ($\bar{M}_n$) was also determined to calculate the polydispersity index ($\bar{M}_w/\bar{M}_n$) of each peak.

Steady-state kinetic studies. The enzymatic rates of MERS-CoV and SARS-CoV PLpro-catalyzed reactions were determined using a modified version of our previously described methods (29, 49). The rates of hydrolysis of a peptide substrate, Z-RLRGG-AMC (Bachem), that contains the C-terminal sequence (RLRGG) of ubiquitin (Ub) and of the full-length Ub and ISG15 substrates Ub-AMC (LifeSensors, Inc.) and ISG15-AMC (Boston Biochem/R&D Systems) were determined by monitoring the increase in fluorescence of the 5-aminomethyl coumarin (AMC) group released (excitation wavelength = 340 nm; emission wavelength = 460 nm) as a function of time. The assays were conducted at 25°C, and the fluorescence was monitored using an EnVision multimode plate reader from PerkinElmer. The initial slope of the reaction, in arbitrary fluorescence units per unit of time (AFU/min), was converted into the amount of hydrolyzed substrate per unit of time (μ M/min) by using a standard curve generated from the fluorescence measurements of well-defined substrate concentrations after complete hydrolysis of the peptide substrates by PLpro to liberate all AMC. All enzymatic reactions were carried out in triplicate. Assays were initiated by the addition of PLpro to assay buffer (50 mM HEPES, pH 7.5, 0.1 mg/ml bovine serum albumin [BSA], 150 mM NaCl, and 2.5 mM DTT). For the Z-RLRGG-AMC assays, 100- μ l reaction mixtures were made in a 96-well black microplate containing various substrate concentrations (50 μ M to 1.6 μ M). The reactions were initiated by the addition of 140 nM SARS-CoV PLpro or 1.6 μ M MERS-CoV PLpro. For Ub-AMC and ISG15-AMC assays, the 30- μ l reaction mixtures were made in half-area, 96-well black microplates from Corning. The Ub-AMC assay used substrate concentrations ranging from 25 μ M down to 0.08 μ M. The reactions were initiated with enzymes to yield final concentrations of 32 nM for SARS-CoV PLpro and 80 nM for MERS-CoV PLpro. The ISG15-AMC assay used various substrate concentrations, from 16 μ M down to 0.03 μ M, and the reactions were initiated with enzymes to yield final concentrations of 1 nM for SARS-CoV PLpro and 6.3 nM for MERS-CoV PLpro. The initial rates of the reactions as a function of substrate concentration were fit to the Michaelis-Menten equation by using the enzyme kinetics module of SigmaPlot (v11; Systat Software Inc.). The resulting steady-state kinetic parameters (k_{cat} and K_M) and their associated errors (Δk_{cat} and ΔK_M) from the fits were then used to calculate k_{cat}/K_M values. The associated error in k_{cat}/K_M values [$\Delta(k_{cat}/K_M)$] was calculated from the following equation; $\Delta(k_{cat}/K_M) = (k_{cat}/K_M)[(\Delta k_{cat}/k_{cat})^2 + (\Delta K_M/K_M)^2]^{1/2}$. When the response of PLpro catalytic activity to increasing substrate concentrations was linear over the substrate concentration range investigated, i.e., when the enzyme could not be saturated

with substrate, the apparent k_{cat}/K_M values were determined by fitting the initial velocity data as a function of substrate concentration, using linear regression.

Enzyme specific activity. To determine enzyme purity and yields during the purification procedure, the specific activity of MERS-CoV PLpro was measured using 250 nM Ub-AMC in assay buffer containing 50 mM HEPES, pH 7.5, 0.1 mg/ml BSA, 150 mM NaCl, and 2.5 mM DTT at 25°C, using the same procedure as that described above.

Protein concentration. The protein concentration during the purification was determined using the cuvette-based Bio-Rad Bradford protein assay.

Inhibition assays and IC_{50} (K_i value) determination. Inhibition of MERS-CoV PLpro by free ubiquitin, free ISG15, and chemical compounds was determined using 30- μ l assay mixtures containing 250 nM Ub-AMC as the substrate and were performed in triplicate, using half-area, 96-well black microplates from Corning. The final enzyme concentrations were 32 nM for SARS-CoV PLpro and 80 nM for MERS-CoV PLpro. The assays were performed at 25°C with increasing concentrations of either free Ub or ISG15, over a range from 55 μ M down to 0.11 μ M. Inhibition assays with compounds were performed at a fixed compound concentration of 100 μ M for known SARS-CoV PLpro inhibitors (28) or 10 μ M for E64. Inhibition assays for HCoV-NL63 PLP2 were performed as described previously (28). Initial rate measurements were determined as described above. Fifty percent inhibitory concentrations (IC_{50} s) for free ubiquitin and ISG15 were determined by plotting the percent inhibition value versus the concentration of inhibitor and then fitting the data using nonlinear regression with the equation $\%I = \%I_{max}/\{1 + (IC_{50}/[I])\}$ and the enzyme kinetics module in the software SigmaPlot (v11; Systat Software Inc.). The resulting IC_{50} s under these experimental conditions are within 5% of the calculated K_i values, which is within experimental error, assuming competitive inhibition (50).

Polyubiquitin chain processing assays. The ability of MERS-CoV and SARS-CoV PLpros to process polyubiquitin chains was determined using assay mixtures containing 50 mM HEPES, pH 7.5, 0.01 mg/ml BSA, 100 mM NaCl, and 2 mM DTT. For SARS-CoV PLpro, a total of 20 nM enzyme was incubated at room temperature with 12 μ g of different ubiquitin chain substrates, including K48-linked Ub₍₄₎, K48-linked Ub₍₅₎, K63-linked Ub₍₆₎, and linear Ub₍₄₎. For MERS-CoV PLpro, a total of 30 nM enzyme was incubated with 6 μ g of K48-linked Ub₍₅₎, K63-linked Ub₍₆₎, and linear Ub₍₄₎. Reaction mixture aliquots of 10 μ l were quenched at different time points after the start of the reaction by using NuPAGE sample buffer (Life Technologies) to a final concentration of 1 $\times$. Identification of the cleaved products was performed on a NuPAGE Bis-Tris gel (Life Technologies) and visualized after staining with Coomassie blue. Each gel was then photographed using a ProteinSimple FluorChem E system. All substrates were purchased from BostonBiochem.

Generation of a MERS-CoV PLpro structural model. Homology models of MERS-CoV PLpro and HCoV-NL63 PLP2 were generated using the structure of SARS-CoV PLpro in complex with ubiquitin aldehyde (PDB entry 4MM3) as the template, as well as the automated Web-based homology modeling server 3D-JIGSAW (Bimolecular Modeling Laboratory, Cancer Research UK, England). Further model refinement was performed using the programs Phenix (51) and Coot (52).

RESULTS

The UBL domain of MERS-CoV nsp3 is not required for the proteolytic, deubiquitinating, or deISGylating activity of PLpro. We previously described the construction of an expression plasmid for a region of nsp3 (residues 1485 to 1802) in MERS-CoV that produced an active form of PLpro capable of catalyzing the *trans*-cleavage of an nsp2/3-GFP substrate in HEK293T cells (40) and deubiquitination and deISGylation of host cell proteins (42). This expression construct contains both the PLpro catalytic and UBL domains (also known as the UB2 domain [53]) of MERS-CoV nsp3, with the addition of 2 amino acids at the N terminus

(methionine and alanine), to allow efficient translation, and a V5 epitope tag on the C terminus, for V5 antibody detection (Fig. 1A). To probe the necessity of the UBL domain for the catalytic function of the PLpro domain in MERS-CoV, we truncated the N terminus by 20, 40, and 60 amino acids (Fig. 1A) within the UBL domain and evaluated the effects of these truncations on the MERS-CoV PLpro protease activity in cell culture. HEK293T cells were transfected with each of the UBL-deleted mutants, along with plasmid DNA expressing the SARS-CoV nsp2/3-GFP substrate (40). Efficient catalytic processing of the nsp2/3-GFP substrate was observed for the full-length wild-type (WT) protein and all UBL-deleted mutants (N20, N40, and N60) (Fig. 1B). In contrast, the MERS-CoV PLpro catalytic cysteine 1594 mutant (CA) was unable to process the substrate, as shown previously (40). These results strongly suggest that the UBL domain of MERS-CoV PLpro is not required for proteolytic processing.

We next tested whether the UBL domain was required for deubiquitination and deISGylation of host cell proteins in cell culture (42). To determine the deISGylating activity of MERS-CoV PLpro constructs, we transfected HEK293T cells with a c-myc-ISG15 plasmid, the ISG15 conjugation machinery, and either MERS-CoV PLpro WT, catalytic mutant, or UBL-deleted mutant proteins. We harvested cell lysates at 18 h posttransfection to evaluate the presence of ISGylated proteins (Fig. 1C). We found that WT PLpro and UBL-deleted mutants deconjugated ISG15 from multiple cellular substrates and that the catalytic cysteine was required for the deconjugation of ISG15. To assess the requirement of the MERS-CoV UBL domain for deubiquitinating activity (DUB) of MERS-CoV PLpro, we transfected HEK293T cells with plasmids expressing Flag-Ub and either MERS-CoV PLpro WT, catalytic mutant, or UBL-deleted mutant proteins. We determined that both WT PLpro and UBL-deleted mutants deubiquitinated multiple cellular substrates and that PLpro catalytic activity was required for DUB activity (Fig. 1D). Together, these data indicate that the UBL domain is not required for the deISGylating and DUB activities of MERS-CoV PLpro.

The UBL domain of MERS-CoV PLpro is not required for its IFN antagonism activity. The observation that the UBL domain of MERS-CoV PLpro is not required for its catalytic activities is consistent with previous studies where it was shown that deletion of the PLpro UBL domain from nsp3 of SARS-CoV did not alter intrinsic proteolytic and DUB activities (44). However, the role of the UBL domain in interferon antagonism is controversial (44, 45). Therefore, we investigated whether MERS-CoV PLpro without the UBL domain can inhibit MDA5-mediated induction of IFN- β . MDA5 has been implicated in recognition of coronaviruses during virus infection (54), and we showed previously that MERS-CoV PLpro with an intact UBL domain functions through this pathway (42). We transfected HEK293T cells with plasmids expressing IFN- β -luciferase, *Renilla* luciferase, pEF-BOS-MDA5 (55), and either the MERS-CoV PLpro WT or catalytic mutant protein at a single concentration or increasing concentrations of UBL deletion mutant N20, N40, or N60. At 16 h posttransfection, we assessed luciferase reporter activity. We determined that MERS-CoV PLpro without its UBL domain can potentially inhibit MDA5-mediated induction of IFN- β in a dose-dependent manner and that catalytic activity is required for IFN- β antagonism (Fig. 2).

Expression and purification of the MERS-CoV PLpro and UBL domains of nsp3. The results of the UBL deletion analysis of

FIG 1 MERS-CoV PLpro constructs, expression, and enzymatic activities in HEK293T cells. (A) MERS-CoV PLpro constructs. Wild-type (aa 1485 to 1802) (WT), catalytic cysteine mutant (Cys1594/A [aa 1485 to 1802]; CA), and three UBL-deleted mutant (N20 [aa 1505 to 1802], N40 [aa 1524 to 1802], and N60 [aa 1545 to 1802]) proteins were fused to a V5 epitope tag at the C terminus for V5 antibody detection. (B) *Trans*-cleavage activity of MERS-CoV PLpro in HEK293T cells expressing SARS-CoV nsp2/3-GFP. Lysates were harvested at 24 h posttransfection, and protein expression was analyzed by Western blotting. DeISGylating (C) and deubiquitinating (D) activities of MERS-CoV PLpro constructs were also examined. HEK293T cells were transfected with MERS-CoV PLpro expression plasmids for WT, CA, and UBL-deleted mutant (N20, N40 or N60) proteins, along with myc-ISG15, E1, E2, and E3 ISGylating machinery plasmids to test the deISGylating (C) activity, or with a Flag-Ub expression plasmid to test the deubiquitinating (D) activity of each PLpro construct. Cells were lysed at 18 h posttransfection and analyzed by Western blotting. The strong bands indicate ISGylated (C) and ubiquitinated (D) proteins. The figure shows representative data from at least two independent experiments.

FIG 2 Interferon antagonism activity of MERS-CoV PLpro. HEK293T cells were transfected with plasmids expressing either wild-type (WT) PLpro, catalytic mutant PLpro (CA), or a UBL-deleted PLpro mutant (N20, N40, or N60). Cells were also transfected with plasmids expressing IFN-luciferase, *Renilla* luciferase, and the stimulator MDA5 (indicated at the top of the figure). At 16 h posttransfection, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate. Error bars represent standard deviations of the means.

MERS-CoV PLpro suggested that we could potentially express and purify a version of MERS-CoV PLpro without its UBL domain. However, we previously attempted to express and purify a version of SARS-CoV PLpro without its UBL domain and found it to be inherently unstable during purification, as it lost catalytic activity over time (56). Therefore, we decided to overexpress and purify MERS-CoV PLpro with its UBL domain intact (residues 1485 to 1802; herein called PLpro) so that we could make a direct comparison with the enzymatic activity of purified SARS-CoV PLpro.

The PLpro domain from MERS-CoV was overexpressed in *E. coli* and purified via three chromatographic steps: (i) Ni^{2+} -charged affinity chromatography followed by removal of the His₈ tag via TEV protease cleavage, (ii) Mono-Q strong-anion-exchange chromatography, and (iii) Superdex 75 size-exclusion chromatography. A summary of the purification procedure, including the enzyme activity yields, fold purification, and resulting specific activities, is presented in Table 1. An SDS-PAGE analysis

TABLE 1 Purification of MERS-CoV PLpro from *E. coli* BL21(DE3)

Sample	Total amt of protein (mg)	Total no. of units (μM/min)	Sp act (μM/mg)	Fold purification	Yield (%)
Lysate	2,625	1,506,335	574	1	100
HisTrap pool	130	1,492,985	11,529	20	99
Mono-Q pool	67	1,095,933	16,309	28	73
Superdex-75 pool	63	1,059,747	16,821	29	70

FIG 3 Purification of MERS-CoV PLpro_{1484–1802}. (A) SDS-PAGE analysis of whole-cell lysate and purified MERS-CoV PLpro, which ran at the expected molecular mass of 37 kDa. Lane M, molecular size marker. (B) SEC-MALS traces of MERS-CoV PLpro at different protein concentrations. MERS-CoV PLpro at 4.2 mg/ml, 2.1 mg/ml, and 1.0 mg/ml eluted at the same retention time from a SEC column. The M_w , determined from the molar mass from the MALS analysis, corresponded to a monomer for the peak of each concentration. All analyzed peak areas were monodispersed (M_w/M_n value of <1.01), as shown by the horizontal traces.

of purified PLpro compared to its expression level in crude lysate is shown in Fig. 3A. The final purified MERS-CoV PLpro enzyme was judged to be over 98% pure. A total yield of 20 mg per liter of cell culture can be obtained by this method. Further experimentation revealed that the addition of a reducing agent (10 mM β ME) and 5% to 10% glycerol is required to avoid protein aggregation during purification and final concentration. The concentrated enzyme was stored at -80°C .

Quaternary structure of MERS-CoV PLpro. We used size-exclusion chromatography coupled with multiangle light scattering (SEC-MALS) to determine the oligomeric state of MERS-CoV PLpro, as well as any potential for aggregation. SEC-MALS analysis revealed an excellent monodispersity of $>90\%$ at the three tested PLpro concentrations (Fig. 3B), in which each sample

eluted at the same retention time. For each peak, the calculated molecular mass was 38.4 ± 3.3 kDa, which is consistent with both the apparent molecular mass (37 kDa) on SDS-PAGE and the expected molecular mass for a monomer (38.1 kDa). These results indicate that MERS-CoV PLpro exists almost exclusively as a monomer in solution, with no detectable higher-order oligomers or aggregates. The unliganded form of SARS-CoV PLpro, on the other hand, tends to form trimers at higher protein concentrations, and it was this form that crystallized with a trimer in the asymmetric unit (49, 56).

Kinetics of hydrolysis of Z-RLRGG-AMC, Ub-AMC, and ISG15-AMC substrates by MERS-CoV and SARS-CoV PLpros. The rates of MERS-CoV PLpro- and SARS-CoV PLpro-catalyzed reactions were examined using three fluorescence-based substrates, including the peptide Z-RLRGG-AMC, which consists of the Ub and ISG15 C-terminal sequences, Ub-AMC, and ISG15-AMC. The kinetic parameters for each coronavirus PLpro and each substrate were determined under the same assay conditions on the same day so that side-by-side experiments could be performed for the most direct comparisons. Due to limitations from inner filter effects produced from the AMC fluorophore at high concentrations of substrate, the assays with Z-RLRGG-AMC were performed at substrate concentrations of no higher than $50 \mu\text{M}$. The kinetic responses of MERS-CoV and SARS-CoV PLpros to increasing concentrations of the 3 substrates are shown in Fig. 4, and the resulting kinetic parameters are summarized in Table 2. As previously observed for SARS-CoV PLpro (57–59), MERS-CoV PLpro exhibited a linear response to increasing substrate concentrations with the peptide substrate Z-RLRGG-AMC (Fig. 4A). Since both enzymes were unable to be saturated with the Z-RLRGG-AMC substrate, we calculated the apparent k_{cat}/K_M values from the slope of the line in Fig. 4A in order to compare their catalytic efficiencies (Table 2). Surprisingly, the activity of MERS-CoV PLpro with the Z-RLRGG-AMC peptide substrate was significantly lower (~ 100 -fold) than that of SARS-CoV PLpro

FIG 4 MERS-CoV and SARS-CoV PLpro activities with three ubiquitin-based substrates. The activities of MERS-CoV PLpro (gray circles) and SARS-CoV PLpro (black circles) with each substrate are shown in panels A to C. Dose-response curves of the inhibition by free Ub and ISG15 are shown in panels D and E. Data were fit to the Michaelis-Menten equation unless the catalytic activity exhibited a linear response to the substrate concentration. In such a case, data were fit to the equation $v/[E] = k_{\text{cat}}/K_M[S]$, where $[E]$ and $[S]$ are the concentrations of enzyme and substrate, respectively. The error bars represent the standard deviations for a minimum of triplicate samples.

TABLE 2 Kinetic parameters and inhibition of PLpro domains from SARS-CoV and MERS-CoV with different substrates

Enzyme	Kinetic parameter	Value with substrate ^c		
		RLRGG-AMC	Ub-AMC	ISG15-AMC
MERS-CoV PLpro	k_{cat}/K_M ($\mu\text{M}^{-1} \text{min}^{-1}$)	0.003 ± 0.0001^a	1.3 ± 0.2	9.9 ± 1.6
	k_{cat} (min^{-1})	—	18.8 ± 1.2	32.6 ± 1.8
	K_M (μM)	—	14.3 ± 2.0	3.3 ± 0.5
	IC_{50} (μM) ^b	—	21.3 ± 4.0	54.4 ± 17.7
SARS-CoV PLpro	k_{cat}/K_M ($\mu\text{M}^{-1} \text{min}^{-1}$)	0.3 ± 0.1^a	1.5 ± 0.3	29 ± 5.3
	k_{cat} (min^{-1})	—	75.9 ± 8.1	436 ± 40
	K_M (μM)	—	50.6 ± 7.4	15.1 ± 2.4
	IC_{50} (μM) ^b	—	NI	18.4 ± 12.2

^a Apparent value derived from the best-fit slope of the data presented in Fig. 4A.^b Values for the inhibition of Ub-AMC hydrolysis by free Ub and free ISG15.^c Values are reported as means $\pm$ standard deviations, based on a minimum of triplicate measurements. —, not determined; NI, no inhibition.

($k_{\text{cat}}/K_M = 0.003 \pm 0.0001 \mu\text{M}^{-1} \text{min}^{-1}$ for MERS-CoV PLpro versus $0.3 \pm 0.1 \mu\text{M}^{-1} \text{min}^{-1}$ for SARS-CoV PLpro). This result suggests that there are significant differences between the enzyme's active sites in terms of recognition and catalysis of the peptide substrate.

In contrast to the case with the Z-RLRGG-AMC peptide substrate, the responses of the PLpro enzymes from both MERS-CoV and SARS-CoV to increasing concentrations of the ISG15-AMC substrate were hyperbolic over a concentration range of 0.03 μM to 16 μM (Fig. 4C). The kinetic response of MERS-CoV PLpro to increasing concentrations of the substrate Ub-AMC was also clearly hyperbolic over a substrate concentration range of 0.08 μM to 25 μM (Fig. 4B). Therefore, the kinetic responses of both MERS-CoV and SARS-CoV PLpros to increasing substrate concentrations were fit to the Michaelis-Menten equation to derive the V_{max} and K_M values, and these values are given in Table 2.

Over a concentration range of 0.08 μM to 25 μM , SARS-CoV PLpro exhibited a curvilinear response to increasing concentrations of Ub-AMC (Fig. 4B). The downward curvature becomes apparent after a concentration of 5 μM , suggesting that the response of SARS-CoV PLpro to Ub-AMC follows Michaelis-Menten kinetics but that the enzyme is still undersaturated at a concentration of 25 μM . Since the initial rate data were obtained in triplicate and the error associated with each measurement was small, we decided to fit the kinetic data to the Michaelis-Menten equation to derive estimates of the kinetic parameters V_{max} and K_M , with the expectation that the errors in these fitted parameters would be higher than those for the other values reported in Table 2. However, the errors in the fitted parameters for SARS-CoV PLpro with Ub-AMC are within the errors associated with V_{max} and K_M for the response of MERS-CoV PLpro with ISG15-AMC and Ub-AMC (Table 2).

The turnover number (k_{cat}) and the catalytic efficiency (k_{cat}/K_M) were calculated for each enzyme (Table 2). Based upon the k_{cat} values, SARS-CoV PLpro catalyzes the turnover of the Ub-AMC and ISG15-AMC substrates approximately 4-fold (75.9 min^{-1} versus 18.8 min^{-1}) and 14-fold (436 min^{-1} versus 32.6 min^{-1}) faster than MERS-CoV PLpro does. SARS-CoV PLpro is also 3 times more efficient than MERS-CoV PLpro in hydrolyzing the ISG15-AMC substrate ($k_{\text{cat}}/K_M = 29 \mu\text{M}^{-1} \text{min}^{-1}$ versus 9.9

$\mu\text{M}^{-1} \text{min}^{-1}$). However, MERS-CoV and SARS-CoV PLpros are equally efficient in hydrolyzing Ub-AMC as a substrate, since their k_{cat}/K_M values are each about $1.3 \mu\text{M}^{-1} \text{min}^{-1}$, due to an ~ 4 -fold equivalent difference between the K_M and k_{cat} values between each enzyme.

In agreement with previous studies using these three substrates (57–59), SARS-CoV PLpro has a significantly higher catalytic efficiency for hydrolysis of the ISG15-AMC substrate than for that of the Ub-AMC (~ 20 -fold) and Z-RLRGG-AMC (~ 100 -fold) substrates. A similar pattern in substrate preference is also observed for MERS-CoV PLpro, as it hydrolyzes the ISG15-AMC (k_{cat}/K_M value of $9.9 \mu\text{M}^{-1} \text{min}^{-1}$) substrate approximately 8 times more efficiently than the Ub-AMC substrate ($k_{\text{cat}}/K_M = 1.3 \mu\text{M}^{-1} \text{min}^{-1}$) and 3,300 times more efficiently than the Z-RLRGG-AMC substrate. Although MERS-CoV and SARS-CoV PLpros exhibit different kinetic parameters for each substrate, they still each prefer a substrate containing ISG15 over Ub.

The most striking kinetic differences between MERS-CoV and SARS-CoV PLpros appear to be in the efficiencies of hydrolysis of the Z-RLRGG-AMC and ISG15-AMC substrates. The origins of the differences for the Z-RLRGG-AMC substrate cannot be ascribed to either k_{cat} or K_M , since we cannot determine these individual kinetic parameters for this substrate. However, the higher activity of SARS-CoV PLpro for ISG15-AMC stems from the more significant differences in the k_{cat} values (436 min^{-1} for SARS-CoV PLpro versus 32.6 min^{-1} for MERS-CoV PLpro) than the K_M values ($15.1 \mu\text{M}$ for SARS-CoV PLpro versus $3.3 \mu\text{M}$ for MERS-CoV PLpro). Interestingly, if one assumes that the K_M values reflect the relative affinities of the enzymes for the substrate, i.e., $K_M = K_d$ (dissociation constant), then both ISG15-AMC and Ub-AMC appear to interact more strongly with MERS-CoV PLpro than with the SARS-CoV PLpro enzyme.

Since K_M values often do not represent the K_d values in enzyme-catalyzed reactions as a result of kinetic complexity, i.e., $K_M \neq k_{-1}/k_1$, we determined the affinities of free ISG15 and Ub for MERS-CoV and SARS-CoV PLpros via steady-state kinetic inhibition studies. Under the experimental conditions utilized, and assuming competitive inhibition, the IC_{50} s determined for ISG15 and Ub are close to the actual K_i values (50). The IC_{50} s for free Ub and ISG15 were therefore determined from a dose-response assay (Fig. 4D and E). The affinity of free Ub for MERS-CoV PLpro ($\text{IC}_{50} = 21.3 \pm 4.0 \mu\text{M}$) is substantially higher than that for SARS-CoV PLpro, since no inhibition was observed for concentrations of up to 60 μM . In contrast, the affinity of free ISG15 for SARS-CoV PLpro ($\text{IC}_{50} = 18.4 \pm 12.2 \mu\text{M}$) is significantly higher than that for MERS-CoV PLpro ($\text{IC}_{50} = 54.4 \pm 17.7 \mu\text{M}$) (Table 2). The differences in IC_{50} s suggest that MERS-CoV PLpro binds Ub significantly more tightly than SARS-CoV PLpro does and that SARS-CoV PLpro binds ISG15 more tightly than MERS-CoV PLpro does. Together, the steady-state kinetic studies suggest that MERS-CoV and SARS-CoV PLpros differ in their abilities to recognize and hydrolyze ubiquitinated and ISGylated substrates.

Recognition and processing of ubiquitin chains by MERS-CoV and SARS-CoV PLpros. Recent X-ray structural and kinetic studies have revealed the complexity behind SARS-CoV PLpro substrate specificity toward polyubiquitin and ISG15 substrates (60). SARS-CoV PLpro was shown to be significantly more active toward K48-linked Ub chains than toward K63-linked Ub chains, as a result of the enzyme possessing a unique bivalent binding site for K48-linked di-Ub chains. Since the molecular structure of

FIG 5 Ubiquitin chain specificity of MERS-CoV and SARS-CoV PLpros. The *in vitro* cleavage of K48-linked Ub₍₅₎ (A) and Ub₍₄₎ (D) by MERS-CoV PLpro and SARS-CoV PLpro, respectively, and of K63-linked Ub₍₆₎ by MERS-CoV PLpro (B) and SARS-CoV PLpro (E) is shown. (C) Cleavage of linear Ub₍₄₎. (F) Analysis of Ub₍₂₎ accumulation during SARS-CoV PLpro-mediated processing of K48-linked substrates. Processing of the substrates is shown by the production of lower-molecular-weight bands at progressive time points, in minutes (') or hours (h). The locations of the different Ub species are shown. Lane M, molecular size marker.

ISG15 resembles that of di-Ub, the preference of SARS-CoV PLpro for ISG15 over Ub is presumed to result from this similarity (60). Therefore, we next examined whether any conservation exists in the abilities of MERS-CoV PLpro and SARS-CoV PLpro to recognize and process K48-linked, K63-linked, or linear polyubiquitin chains.

MERS-CoV and SARS-CoV PLpros, at a concentration of 1.6 nM, were first incubated overnight with 1 μ g each of the Ub-based substrates: K48-linked Ub₍₅₎, K63-linked Ub₍₆₎, and linear Met1-Ub₍₄₎. Analysis of the reaction products by SDS-PAGE indicated that only SARS-CoV PLpro was capable of processing the K48-linked Ub₍₅₎ and K63-linked Ub₍₆₎ substrates under these conditions, as few to no reaction products were observed with the MERS-CoV PLpro reactions (data not shown). The low activity of MERS-CoV PLpro was the first indication that the enzyme has poorer catalytic activity toward polyubiquitin chains than that of the SARS-CoV PLpro enzyme. Therefore, in order to detect any patterns in the products cleaved by MERS-CoV PLpro, the PLpro enzyme concentration was increased to 5 nM and the reaction products were analyzed over a period of 18 h by SDS-PAGE (Fig. 5). Over the first 1 h of the reaction of MERS-CoV PLpro with both K48-linked Ub₍₅₎ and K63-linked Ub₍₆₎ substrates, the accumulation of lower-molecular-weight ubiquitin chain products was apparent (Fig. 5A and B). We observed no significant differences in the debranching patterns or processing rates of K48- versus K63-linked substrates by MERS-CoV PLpro over a 1-h period, and after 18 h, the reactions were almost complete. Neither the MERS-CoV nor SARS-CoV PLpro enzyme was able to hydrolyze linear Ub₍₄₎ (Fig. 5C).

The processing of K48-linked Ub₍₅₎ and K63-linked Ub₍₆₎ substrates by MERS-CoV PLpro ultimately resulted in the formation of a mono-Ub species after 18 h. SARS-CoV PLpro, on the other

hand, hydrolyzed K48-linked Ub₍₅₎ (Fig. 5F) significantly faster than K63-linked Ub₍₆₎ (Fig. 5E). Moreover, SARS-CoV hydrolysis of K48-linked Ub led to the accumulation of di-Ub products over time (Fig. 5D and F), whereas hydrolysis of the K63-linked Ub₍₆₎ substrate was much slower and did not lead to the accumulation of di-Ub species. Because SARS-CoV PLpro has a higher affinity for K48-linked di-Ub molecules (60), the accumulation of K48-linked di-Ub in the Sub2 and Sub1 binding subsites leads to product inhibition by lowering the rate of debranching of the longer K48-linked Ub chains or the further cleavage of di-Ub into mono-Ub. This phenomenon is better observed during the processing of polyubiquitin chains with an even number of ubiquitins, such as K48-linked Ub₍₄₎. With this substrate, little to no mono-Ub was produced during the course of the reaction (Fig. 5D), whereas cleavage of K48-linked Ub₍₅₎ produced Ub₍₄₎, Ub₍₃₎, Ub₍₂₎, and mono-Ub over time (Fig. 5F). However, for MERS-CoV PLpro, debranching of K48-linked polyubiquitin chains with an even or odd number of ubiquitins resulted in an increase of mono-Ub. These results support a model whereby MERS-CoV PLpro does not interact with K48-linked polyubiquitin chains via a bivalent recognition mechanism, as does SARS-CoV PLpro (60). Therefore, recognition of polyubiquitin chains by MERS-CoV PLpro occurs primarily through a monovalent Ub interaction, presumably within the zinc finger and palm regions of the enzyme.

Inhibitors of SARS-CoV PLpro and HCoV-NL63 PLP2 do not inhibit MERS-CoV. Our most recent effort toward the development of SARS-CoV PLpro inhibitors generated a new series of competitive inhibitors with significant improvements toward the development of anti-SARS drugs (28). These newer inhibitors have improved inhibitory potency and SARS-CoV antiviral activity, better metabolic stability, and lower cytotoxicity than those of our previous generations of inhibitors. Furthermore, none of the

FIG 6 MERS-CoV PLpro and HCoV-NL63 PLP2 inhibition by a series of SARS-CoV PLpro inhibitors. The percent inhibition of SARS-CoV PLpro, HCoV-NL63 PLP2, and MERS-CoV PLpro activity in the presence of SARS-CoV PLpro inhibitors is shown by a graph. Percent inhibition was calculated from two independent assays with a fixed concentration of 100 μ M compound, and data are shown as mean % inhibition. Error bars representing the positive and negative deviations from the average values were removed for clarity. The difference between each independent measurement was less than 10% for the entire set of data. Highlighted in bold are the best SARS-CoV PLpro inhibitor candidates, including compound 3k, also shown in Fig. 7C. (Inset) Chemical structure of compound 3k.

compounds show off-target inhibitory activity toward a number of human DUB enzymes or cysteine proteases. Interestingly, a number of the compounds also show inhibitory activity against the PLP2 catalytic domain of nsp3 from HCoV-NL63, providing a basis for the potential development of broader-spectrum inhibitors against various CoV PLpro domains. Therefore, we tested whether any of these compounds have the ability to inhibit the enzymatic activity of MERS-CoV PLpro. The inhibitory activity of 28 compounds was tested against MERS-CoV PLpro, SARS-CoV PLpro, and HCoV-NL63 PLP2, and the data are shown in Fig. 6. Surprisingly, even though both SARS-CoV PLpro and MERS-CoV PLpro belong to group 2 coronaviruses and share significant amino acid sequence homology ($\sim 50\%$ homology), no significant inhibition of MERS-CoV PLpro was observed for any of the compounds at a concentration of 100 μ M. In contrast, HCoV-NL63 PLP2 is from the more distantly related group 1 coronaviruses and shares only about 30% homology with SARS-CoV PLpro, yet it was inhibited by over half of the compounds, 10 of which produced $>50\%$ inhibition. These results suggest that a low level of sequence conservation may exist between inhibitor-binding sites that is not necessarily related to the coronavirus group specification and that subtle structural differences may be significant determinants in attempting to develop broad-spectrum inhibitors against CoV PLpro enzymes. In support of this hypothesis, we found that E64, a cysteine protease inhibitor that reacts covalently with the active site cysteine of proteases, exclusively inhibited HCoV-NL63 PLP2, not MERS-CoV or SARS-CoV PLpro, suggesting that the binding site near the active site cysteine is not highly conserved among these PLpros.

Homology model of MERS-CoV PLpro. To gain insight into the structural differences between MERS-CoV and SARS-CoV PLpros that may elicit the differences in their substrate and inhibitor specificities, we generated an energy-minimized molecular model of MERS-CoV PLpro based on the available structures of SARS-CoV PLpro (Fig. 7). The homology model was built and refined against the electron density of SARS-CoV PLpro in complex with Ub aldehyde (PDB entry 4MM3) (60). The resulting structural model of MERS-CoV PLpro was analyzed by overlaying it with the structures of SARS-CoV PLpro in complex with Ub and

the inhibitor 3k (28). The domains of SARS-CoV PLpro (aa 1541 to 1884) and MERS-CoV PLpro (aa 1484 to 1802) share 52% overall homology. During model refinement, we examined the substrate/inhibitor-binding domain at the enzyme subsites in the palm domain, the oxyanion hole, and the ridge region (60) of the thumb domain (Fig. 7A). The resulting and refined homology model was then compared to the recently reported X-ray crystal structure of unliganded MERS-CoV PLpro (61). The structures were found to be very similar, with the exception of the active site loop, which is missing in the X-ray structure as a result of no observable electron density. More details of the comparison, especially around the active site loop, can be found in Fig. 8. Since our homology structure coincided closely with the X-ray structure and since our structure contains an energy-minimized model of the active site loop, we continued our analysis with the homology model and indicate any major differences with the X-ray structure, which were few in the structural regions of interest.

The X-ray crystal structure of SARS-CoV PLpro in complex with Ub-aldehyde revealed that the majority of PLpro-Ub interactions occur between PLpro and the five C-terminal (RLRGG) residues of Ub (60, 62). Therefore, we examined the amino acid conservation at the enzyme subsites of MERS-CoV PLpro. We predict that only 8 of 12 hydrogen bonds (H bonds) are likely to be conserved in the MERS-CoV PLpro-Ub C terminus interactions, among which 5 H bonds occur between Ub and the backbone of PLpro (Fig. 7B). The loss of 4 H bonds is due to the nonconserved changes of E168, Y265, and W107 from SARS-CoV PLpro to R170, F271, and L108, respectively, in MERS-CoV PLpro. These predictions are in agreement with the kinetic studies, which showed that SARS-CoV PLpro is 100-fold more active than MERS-CoV PLpro with the peptide substrate Z-RLRGG-AMC (Table 2). Therefore, unlike SARS-CoV PLpro, in which the Ub C terminus provides a significant energetic contribution of binding, for MERS-CoV PLpro, greater binding energy is likely provided by interactions outside the Ub C-terminal RLRGG residues.

Other potential amino acid differences within the enzyme subsites may also explain the lack of inhibition by compounds designed to be inhibitors of SARS-CoV PLpro. A structural alignment of the MERS-CoV PLpro homology model with the X-ray

FIG 7 Analysis of MERS-CoV PLpro subsites, active site, and ridge region of the thumb domain. (A) Homology model of MERS-CoV PLpro (gray surface, yellow cartoon), displaying the canonical right-hand architecture, with thumb, palm, and zinc finger domains, and with an additional UBL domain at the N terminus. Modeled Ub (pink) is positioned onto the Ub-binding domain in the zinc finger with its C terminus extending into the active site. The areas highlighted with boxes are the regions of the thumb domain and palm domain predicted to be responsible for MERS-CoV PLpro divergence from SARS-CoV PLpro substrate and inhibitor specificities. (B) Enzyme subsites, displaying the predicted intermolecular interactions with the Ub C terminus. Green dashed lines indicate the H bonds between SARS-CoV PLpro (blue cartoon) and the Ub C terminus that are predicted to be conserved in MERS-CoV PLpro. The black dashed lines indicate H bonds or salt bridges that are predicted to be lost in the MERS-CoV PLpro–Ub C terminus interaction. Amino acids involved in SARS-CoV PLpro–Ub C terminus interactions are shown in blue font, and the predicted corresponding amino acids in MERS-CoV PLpro are shown in black font. Residues highlighted in bold are the nonconserved amino acid substitutions in MERS-CoV PLpro. (C) SARS-CoV PLpro in complex with compound 3k (orange ball-and-stick model) (PDB entry 4OW0), overlaid on MERS-CoV PLpro and a homology model of HCoV-NL63 PLP2 (green). The amino acid residues important for SARS-CoV PLpro–inhibitor interactions are shown (blue font), along with the predicted corresponding amino acids in HCoV-NL63 PLP2 (green font) and MERS-CoV PLpro (black font). The residues highlighted in bold are the nonconserved substitutions in MERS-CoV PLpro. At the bottom of panel C is a comparison between SARS-CoV, HCoV-NL63, and MERS-CoV PLpro amino acid compositions of the β-turn/loop (highlighted with an arrow), known to be important for the inhibitor-induced-fit mechanism of association of compound 3k and SARS-CoV PLpro. (D) Comparison of the active site and oxyanion hole, showing the corresponding amino acids in SARS-CoV, HCoV-NL63, and MERS-CoV PLpros. (E) Overlay of SARS-CoV PLpro and MERS-CoV PLpro ridge regions of the thumb domain. Amino acid numbering is defined as follows: for SARS-CoV PLpro, aa 1 corresponds to aa 1540 in the polyprotein; for HCoV-NL63 PLP2, aa 1 corresponds to aa 1578 in the polyprotein; and for MERS-CoV PLpro, amino acid 1 corresponds to amino acid 1480 in the polyprotein.

structure of SARS-CoV PLpro in complex with the inhibitor 3k (28), depicting the amino acid residues involved in SARS-CoV PLpro–inhibitor binding, is shown in Fig. 7C. Because SARS-CoV PLpro inhibitors can also inhibit the PLP2 domain from HCoV-NL63, a homology model of HCoV-NL63 PLP2, constructed via the same approach as that used for MERS-CoV PLpro, is included for comparison in Fig. 7C. From these two structural models, we predict that a number of amino acid differences between the enzymes occur within the hydrophobic pocket comprising P248–P249 and at the flexible β-turn/loop (BL2 loop or Gly267–Gly272), known to participate in an induced-fit mechanism of inhibitor association (28). Modeling of the β-turn/loop of MERS-CoV PLpro was significantly challenging due to the presence of an additional amino acid, therefore rendering a longer loop with absolutely no amino acid conservation to SARS-CoV PLpro. On the other hand, more conserved substitutions are predicted for

HCoV-NL63 PLP2, in which Y269 and Q270, both important for binding of compound 3k (28), are replaced by F255 and D265, respectively. Another important difference is observed at the entrance of the active site, where L163 in SARS-CoV PLpro acts as a gatekeeper at the S subsites, blocking access to the catalytic triad (56). Upon inhibitor binding, L163 folds backwards, accommodating the substituted benzamide groups of the inhibitors (28–30). For HCoV-NL63 PLP2, this amino acid is replaced by K152, but in MERS-CoV PLpro, the less conserved replacement by P165 at this position might render the entrance to the active site much more rigid and therefore unable to accommodate inhibitor substituents.

Since bulky or rigid amino acid residues at the S subsites hinder access to the active site and catalytic cysteine, we then examined the oxyanion hole at the S' subsites of HCoV-NL63 PLP2 as a possible inhibitor-binding site for the covalent cysteine protease

FIG 8 Comparison between MERS-CoV PLpro β -turn region and enzyme subsites identified via molecular modeling and the recently reported X-ray crystal structure. A structural superposition between the refined homology model of MERS-CoV PLpro (yellow cartoon) and the recently reported X-ray crystal structure (PDB entry 4P16; green cartoon), which was reported during review of the manuscript, yields a C- α root mean square deviation (RMSD) value of 2.1 Å for 268 atoms aligned. The $2F_o - F_c$ electron density map from PDB entry 4P16 is contoured at 1σ (shown as gray mesh) and confirms the presence and locations of the amino acids predicted for the enzyme subsites by the structural model (labeled amino acids; shown as sticks). The residues comprising the β -turn in the 4P16 structure are missing in the X-ray structure due to the lack of associated electron density. The refined homology model contains this loop region and therefore serves as a useful structural model for understanding the interactions between the loop and substrates or inhibitors. The striking similarity between the X-ray crystal structure and our energy-minimized structural model demonstrates the high quality of our computational analyses and makes it a good model for predicting a potential conformation for the β -turn of MERS-CoV PLpro.

inhibitor E64 (Fig. 7D). We found that the oxyanion hole of HCoV-NL63 PLpro is occupied by the small amino acid T96, in contrast to the bulky oxyanion hole residues W107 and L108, found in SARS-CoV PLpro and MERS-CoV PLpro, respectively. The presence of a smaller amino acid residue in the oxyanion hole of HCoV-NL63 PLP2 could render a larger cavity at the S' subsites of the enzyme, thus explaining why E64 can only form a covalent adduct on the catalytic cysteine of HCoV-NL63 PLP2.

We have shown that MERS-CoV PLpro does not share SARS-CoV PLpro substrate specificity at the SUB2 site for distal Ub molecules. Therefore, we examined the amino acid conservation at the ridge region of the thumb domain, which is the site in SARS-CoV PLpro responsible for the SUB2-Ub interaction (60). In our homology model, we find very low amino acid conservation at the ridge of the thumb domain. Moreover, the model suggests that a longer helix $\alpha 2$ (56) may exist at the SUB2 site (Fig. 7E). Therefore, the lack of conservation between the MERS-CoV PLpro and SARS-CoV PLpro ridge regions of the thumb domain can explain why MERS-CoV PLpro cannot interact with Ub/UBL modifiers with a bivalent mechanism of binding.

DISCUSSION

The papain-like protease (PLpro) domains of coronavirus nsp3's are monomeric enzymes that perform multiple cellular functions to facilitate viral replication (reviewed in reference 39). Among these functions is the essential role of recognizing and processing the viral replicase polyprotein at the boundaries of nsp1/2, nsp2/3, and nsp3/4 (27, 40, 41, 63). Other physiological roles of CoV PLpros are less understood but involve the removal of Ub (deubiquitination) and the ubiquitin-like modifier ISG15 (deISGylation) from cellular proteins. The global removal of ISG15 and ubiquitin from numerous host cell proteins has been shown to interfere with the production of type 1 interferon (IFN), which facilitates viral evasion from the host's antiviral defenses (64). So far, the multifunctionality of PLpro domains within nsp3 appears to be a conserved feature among CoVs, as at least one of the two encoded PLpro domains, typically PLP2, has isopeptidase activity (43–45, 48, 57). However, SARS-CoV and MERS-CoV, which belong to *Betacoronavirus* group 2, encode only one PLpro domain within nsp3, which is an ortholog to the PLP2 domain from other CoVs encoding two PLpro domains. Although CoV PLpros catalyze the same chemical reaction, i.e., hydrolysis of peptide and isopeptide bonds, recent structural and kinetic studies on the substrate specificities of SARS-CoV PLpro demonstrate the uniqueness of SARS-CoV PLpro among other CoV PLpros studied so far in terms of recognizing and processing ubiquitin chains (60, 62). These studies and the ones reported here for MERS-CoV PLpro suggest that even the most closely related orthologs can differ significantly in terms of substrate recognition, enzymatic activity, and inhibition by small-molecule compounds. Such differences emphasize the importance of investigating in detail the biochemical reaction mechanisms in conjunction with cellular activities to gain a better understanding of how CoV PLpros conduct their multifunctional roles.

The steady-state kinetic characterization of MERS-CoV PLpro and SARS-CoV PLpro revealed differences among their substrate preferences. Recent X-ray structural analyses of SARS-CoV PLpro in complex with Ub show that the C-terminal amino acids RLRGG of ubiquitin occupy the S4-S1 enzyme subsites of SARS-CoV PLpro (60, 62). These interactions appear to provide a significant amount of the total binding energy for stabilization of the PLpro-Ub complex by formation of 12 intermolecular H bonds that result from substrate-induced conformational rearrangement of the flexible β -turn/loop (60, 62), also called the BL2 loop (56) or the $\beta 14$ - $\beta 15$ loop (62). The S4-S2 subsites are also the binding sites for SARS-CoV PLpro competitive inhibitors, and similar to the case for substrate binding, the flexible β -turn/loop adopts a conformational change to allow for optimal inhibitor interactions (28–30). In contrast, we found that MERS-CoV PLpro behaves significantly differently from SARS-CoV PLpro in terms of recognition and hydrolysis of the Ub/ISG15 C terminus-based substrates and Z-RLRGG-AMC, as well as inhibition by SARS-CoV PLpro inhibitors. The activity of MERS-CoV PLpro toward the Z-RLRGG-AMC substrate is 100-fold lower than that with SARS-CoV PLpro (Fig. 4A; Table 2), suggesting that the enzymes differ in substrate recognition at the subsites. Analysis of the amino acid conservation in the predicted S4-S1 subsites of MERS-CoV PLpro indicates low sequence conservation, which might lower the available number of intermolecular H bonds between the MERS-CoV PLpro active site and the Ub C-terminal

residues (Fig. 7B). The net effect of these sequence differences may perhaps reduce the affinity of the Z-RLRGG-AMC substrate with the MERS-CoV protein and/or lower the catalytic activity.

Additional support for differences in molecular recognition between SARS-CoV and MERS-CoV PLpros comes from the fact that the numerous SARS-CoV PLpro inhibitors tested here do not inhibit MERS-CoV PLpro (Fig. 6). The lack of inhibition of MERS-CoV PLpro by these inhibitors most likely stems from the structural differences between the S4-S1 subsites, which are revealed via comparison of the MERS-CoV PLpro homology model and SARS-CoV X-ray structures (Fig. 7). Noteworthy structural differences are observed at the flexible β -turn/loop, which in MERS-CoV PLpro is one residue longer than SARS-CoV PLpro (Fig. 7C). A comparison of the amino acids within the β -turns/loops (between the flanking glycine residues) among the different human and animal CoVs indicates little to no conservation (Fig. 9). One notable exception is HCoV-NL63 PLP2, which is moderately inhibited by SARS-CoV PLpro inhibitors (Fig. 6) (28). HCoV-NL63 PLP2 has the same number of residues within the β -turn/loop and also has a phenylalanine (F255) in a position equivalent to that of the tyrosine residue (Y269) in SARS-CoV PLpro that interacts with inhibitors (Fig. 7C). Based on the low amino acid sequence conservation within the β -turn/loop among the PLpros, we predict that this series of inhibitors is unlikely to be effective against the other clinically relevant HCoVs, including 229E-CoV, which has the same number of amino acids; MERS-CoV, which has an extra amino acid; and HKU1 and OC43, which have shorter β -turns/loops, by one amino acid (Fig. 9). Similarly, these predictions apply to CoVs from animals, such as bovine CoV (BCoV), porcine hemagglutinating encephalomyelitis virus (PHEV), porcine respiratory coronavirus (PRCV), transmissible gastroenteritis virus (TGEV), and feline and canine CoVs (FCoV and CCoV).

The Ub and UBL modifier specificities of many viral and human DUB enzymes depend strongly on the type of polyubiquitin linkage, the chain length, and the number of Ub-interacting domains encoded in the structure of the enzyme (65–68). Moreover, it is well established that the great topological diversity postulated by 8 different types of polyubiquitin chains provides additional regulatory elements of Ub recognition by DUB enzymes (65). We show through the studies reported here that the MERS-CoV PLpro substrate specificity for Ub/UBL modifiers differs from that of SARS-CoV PLpro. MERS-CoV PLpro can interact more strongly than SARS-CoV PLpro with mono-Ub substrates, but its polyubiquitin chain debranching activities toward K48-linked and K63-linked polyubiquitin substrates are less robust than those of SARS-CoV PLpro. MERS-CoV PLpro is able to process both K48- and K63-linked substrates equally well, converting

both substrates into mono-Ub species over time (Fig. 5A and B). SARS-CoV PLpro, on the other hand, has reduced activity toward K63-linked polyubiquitin chains compared to K48-linked polyubiquitin chains (Fig. 5), and its activity toward ISG15-linked substrates is higher than that of any DUB or de-ISGylating enzyme studied to date (59, 60).

Unlike MERS-CoV PLpro, SARS-CoV PLpro loses its ability to rapidly cleave K48-linked polyubiquitin chains over time, due to the accumulation of di-Ub reaction products (Fig. 5). We recently demonstrated that this phenomenon of product inhibition stems from the fact that SARS-CoV PLpro prefers to bind K48-linked di-Ub molecule chains via a bivalent interaction with the enzyme's zinc finger domain and the ridge region of the thumb domain (Fig. 10). The two Ub-interacting sites are designated SUB1 (at the zinc finger) and SUB2 (at the ridge region of the thumb domain). These two distal Ub/UBL subsites are capable of interacting simultaneously with K48-linked di-Ub and ISG15 but not K63-linked polyubiquitin chains, which are topologically different. Due to the greater affinity of K48-linked di-Ub for the SARS-CoV PLpro enzyme, the accumulation of the di-Ub reaction product during chain processing results in product inhibition (60). With an even number of K48-linked ubiquitins in the polyubiquitin chain, e.g., with Ub₍₄₎, we observed an even greater accumulation of the di-Ub species over time with SARS-CoV PLpro (Fig. 5D) than the case with K48-linked polyubiquitin chains with an odd number of Ub moieties, which produce both mono-Ub and di-Ub (Fig. 5F). In contrast, MERS-CoV PLpro does not show a build-up of di-Ub in its processing of any polyubiquitin chain, suggesting that it does not contain an Sub2 site on the MERS-CoV PLpro enzyme surface.

The lack of amino acid conservation at the predicted Sub2 site (Fig. 7E and 9) may be the reason for the polyubiquitin chain processing differences between MERS-CoV and SARS-CoV PLpros. Analysis of the amino acid sequence conservation at the ridge region of the thumb domain among all CoV PLpros shows very little conservation, suggesting that the bivalent recognition of K48-linked Ub₍₂₎ may be a unique feature of SARS-CoV PLpro (Fig. 9). However, since the majority of CoV PLpros have not yet been characterized fully in terms of their polyubiquitin chain recognition and processing activities, more research is required to better understand the potential implications of different polyubiquitin recognition patterns during the PLpro-mediated antagonism of the innate immune response and how differences in recognition can affect the pathogenicity of these human coronaviruses.

In Fig. 10, we propose a general model describing the mechanisms of chain processing of K48-linked Ub₍₄₎ by SARS-CoV PLpro and MERS-CoV PLpro. For SARS-CoV PLpro, processing

FIG 9 Multiple-sequence alignment, generated with ESript, presenting the secondary structure elements on top, as follows: squiggles, α -helices; black arrows, β -strands; and TT, turns. Highlighted are the highly conserved areas (blue outlined boxes) containing the conserved residues (red filled boxes), homologous residues (red font), and divergent residues (black font). The structural elements were generated using the X-ray crystal structure of SARS-CoV apo-PLpro (PDB entry 2FE8). MERS-CoV PLpro UBL truncation sites N20, N40, and N60 are marked in purple, and the catalytic triad residues are highlighted with asterisks. α -Helix 2 (highlighted with a green box), containing the amino acid residues important for SARS-CoV PLpro interaction with K48-Ub₂ and ISG15, is highly divergent among CoV PLpros. The amino acid residues important for interactions with SARS-CoV PLpro inhibitors are highlighted with a blue filled box. The β -turn/loop at the inhibitor-binding site (highlighted with a black outlined box) is highly divergent among CoV PLpros. Sequence accession numbers are as follows: SARS-CoV PLpro_{21541–1854}, [AAP13442.1](#); HCoV-NL63 PLP_{21578–1876}, [YP_003766.2](#); MERS-CoV PLpro_{1484–1802}, [AFS88944.1](#); HCoV-HKU1 PLP2_{1648–1955}, [YP_173236](#); HCoV-OC43 PLP2_{1562–1870}, [CAA49377.1](#); HCoV-229E PLP2_{1599–1905}, [CAA49377.1](#); PHEV-CoV PLP2_{1561–1871}, [YP_459949.1](#); PRCV-CoV PLP2_{1484–1780}, [DQ811787](#); TGEV-CoV PLP2_{1487–1783}, [CAA83979.1](#); FCoV PLP2_{1441–1920}, [AAY32595](#); CCoV PLP2_{1441–1920}, [AFX81090](#); BCoV PLP2_{21562–1870}, [NP_150073](#); and MHV-A59 PLP2_{1606–1915}, [NP_068668.2](#).

FIG 10 Model for the processing of K48-linked Ub₍₄₎ by SARS-CoV PLpro and MERS-CoV PLpro. Schematic diagrams show two mechanisms for the recognition of the distal Ub (B and C) from K48-linked Ub₍₄₎ (A). (B) The distal Ub-interacting subsites SUB1 and SUB2 are shown for a bivalent mode of recognition, with one Ub subsite at the zinc finger and the second Ub subsite at the ridge region of the thumb domain. (C) The monovalent mechanism of distal Ub recognition uses only the SUB1 site at the zinc finger. The position of the substrate's scissile bond in the active site is indicated with a red arrow, and the reaction progress is shown as product accumulations 1, 2, and 3. (D) SARS-CoV PLpro has a bivalent mode of recognition toward K48-linked polyubiquitin chains (mechanism 1) and has a high affinity for K48-linked di-Ub molecules. In the case of K48-linked Ub₍₄₎, the first cleavage event occurs through the bivalent interaction of the SARS-CoV PLpro zinc finger and ridge region of the thumb domain with di-Ub, producing two di-Ub cleavage products. Subsequent cleavage events occur much more slowly due to the less favorable binding of mono-Ub than di-Ub molecules. (E) MERS-CoV PLpro interacts with K48-linked polyubiquitin chains via a monovalent mode of recognition (mechanism 2) and has a moderate affinity for mono-Ub molecules. Cleavage of K48-linked Ub₍₄₎ occurs through the monovalent interaction of the MERS-CoV PLpro zinc finger with mono-Ub, with no significant differences in the rates of processing tetra-, tri-, and di-Ub species. Other possible cleavage routes are shown with blue arrows.

begins with the bivalent recognition and interaction of two Ub molecules (Ub1 and Ub2) in a K48-linked polyubiquitin chain at SUB1 in the zinc finger and SUB2 in the ridge region of the thumb domain (Fig. 10B and D). The endo-trimming of the isopeptide bond between Ub1, bound at the SUB1 subsite, and Ub1', bound at the SUB1' subsite, results in the overall production of a di-Ub molecule and a single Ub molecule from a Ub₍₃₎ chain, a second di-Ub molecule from a Ub₍₄₎ chain, and a Ub₍₃₎ chain from a Ub₍₅₎ chain, which can be processed further to di-Ub and mono-Ub

molecules. In order for SARS-CoV PLpro to further cleave the di-Ub molecules to mono-Ub, di-Ub has to be released from the enzyme (product release), which appears to be the slow step in the kinetic mechanism of K48-polyubiquitin chain processing. For MERS-CoV PLpro, however, since there is no detectable accumulation of reaction products over time (Fig. 5A and B), and because mono-Ub has a moderate affinity for the enzyme (Fig. 4D and Table 2), processing occurs in a stepwise manner, with equal opportunities for endo- and exo-trimming of the chain (Fig. 10C

and E). As a result, by trimming polyubiquitin chains via its Sub1 subsite, there is no substantial difference in the rates of processing of different lengths of K48-linked chains.

So far, few studies have been reported on the specificity of SARS-CoV PLpro beyond the P1' position of the substrate. It has been demonstrated only that SARS-CoV PLpro is able to cleave peptide substrates containing the P1' amino acid residues Ala, Gly, Asp, and Lys (69). Surprisingly, even though CoV PLpros can cleave the peptide bonds within the polyprotein cleavage sites and hydrolyze AMC from Ub-AMC and ISG15-AMC, neither the MERS-CoV nor SARS-CoV PLpro enzyme is able to hydrolyze the peptide bond from Met1-linked linear Ub₍₄₎ (Fig. 5C). The cleavage site for linear ubiquitin would be R-L-R-G-G|M-Q-I-F-V. The lack of cleavage activity with a Met1-linked polyubiquitin chain indicates either that the S1' subsites of PLpros cannot accommodate the bulky side chain of Met at the P1' position or that the amino acids Q, I, F, and V at P2', P3', P4', and P5' may prevent cleavage. It is clear that PLpro enzymes do not have specificity for linear polyubiquitin chains.

In summary, the substrate, inhibitor, and ubiquitin chain recognition patterns of PLpros from MERS-CoV and SARS-CoV are similar, with SARS-CoV PLpro having more robust catalytic activity toward most substrates and exhibiting a unique bivalent recognition mechanism toward polyubiquitin substrates. Both enzymes are capable of recognizing and hydrolyzing fluorophores from the C termini of RLRGG peptide, Ub, and ISG15 substrates, yet the kinetic parameters associated with these reactions are different. Neither enzyme is capable of cleaving the peptide bond between two Ub molecules within a Met1-linked polyubiquitin chain, but both enzymes are capable of recognizing and cleaving K48-linked and K63-linked polyubiquitin chains. Our detailed analysis revealed that MERS-CoV PLpro prefers to recognize and bind a single Ub molecule within its Sub1 subsite, allowing it to perform either endo- or exo-trimming of K48- and K63-linked polyubiquitin chains, whereas SARS-CoV PLpro performs such trimming only on K63-linked chains, and does so slowly. We also found that SARS-CoV PLpro utilizes a unique bivalent recognition mechanism for K48-linked polyubiquitin chains, whereby it binds two ubiquitin molecules in the Sub1 and Sub2 subsites and performs mainly endo-trimming reactions releasing di-Ub. The ramifications of these ubiquitin chain preferences on the innate immune response during coronavirus infection should be explored. Indeed, using structure-guided mutagenesis, we diminished the ability of SARS-CoV PLpro to preferentially bind di-Ub and ISG15 over mono-Ub, which caused a significant decrease in the ability to stimulate the NF- κ B pathway (60). These results suggest that subtle differences in polyubiquitin chain cleavage specificity may have functional ramifications for viral pathogenesis.

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