The Papain-Like Protease of Severe Acute Respiratory Syndrome Coronavirus Has Deubiquitinating Activity

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Replication of the genomic RNA of severe acute respiratory syndrome coronavirus (SARS-CoV) is mediated by replicase polyproteins that are processed by two viral proteases, papain-like protease (PLpro) and 3C-like protease (3CLpro). Previously, we showed that SARS-CoV PLpro processes the replicase polyprotein at three conserved cleavage sites. Here, we report the identification and characterization of a 316-amino-acid catalytic core domain of PLpro that can efficiently cleave replicase substrates in trans-cleavage assays and peptide substrates in fluorescent resonance energy transfer-based protease assays. We performed bioinformatics analysis on 16 papain-like protease domains from nine different coronaviruses and identified a putative catalytic triad (Cys1651-His1812-Asp1826) and zinc-binding site. Mutagenesis studies revealed that Asp1826 and the four cysteine residues involved in zinc binding are essential for SARS-CoV PLpro activity. Molecular modeling of SARS-CoV PLpro suggested that this catalytic core may also have deubiquitinating activity. We tested this hypothesis by measuring the deubiquitinating activity of PLpro by two independent assays. SARS CoV-PLpro hydrolyzed both diubiquitin and ubiquitin–7-amino-4-methylcoumarin (AMC) substrates, and hydrolysis of ubiquitin-AMC is approximately 180-fold more efficient than hydrolysis of a peptide substrate that mimics the PLpro replicase recognition sequence. To investigate the critical determinants recognized by PLpro, we performed site-directed mutagenesis on the P6 to P2' residues at each of the three PLpro cleavage sites. We found that PLpro recognizes the consensus cleavage sequence LXGG, which is also the consensus sequence recognized by cellular deubiquitinating enzymes. This similarity in the substrate recognition sites should be considered during the development of SARS-CoV PLpro inhibitors.
viral agents that inhibit SARS-CoV replication (2, 7, 59, 60). In contrast, no structural information is currently available for any coronavirus PLpro. Molecular modeling of a HCoV-229E papain-like protease suggested that a zinc-binding domain, which connects the left- and right-hand domains of a papain-like fold, might be important for protease activity and that protease activity is mediated by a cysteine-histidine catalytic dyad (19). Interestingly, many coronaviruses encode two functional papain-like proteases, termed PLP1 and PLP2 (or PL1pro and PL2pro). At least two coronaviruses, SARS-CoV and aIBV, encode only one functional papain-like protease, which we term PLpro. The coronavirus papain-like proteases have been shown to process the amino-terminal end of the replicase polyprotein to generate two or three replicase products (3, 4, 8, 17, 18, 25, 35), but the exact role of proteolytic processing in the viral replication cycle has not yet been elucidated.

For SARS-CoV, the PLpro domain is contained within nsp3, a 213-kDa membrane-associated replicase product. Previously, we cloned and expressed a 73-kDa SARS-CoV PLpro domain and developed a trans-cleavage assay to assess protease activity. We found that SARS-CoV PLpro can function in trans and that it is responsible for processing nsp1, nsp2, and nsp3 from the amino-terminal end of the replicase polyprotein (17). In addition, a recent report by Sulea and colleagues predicted that SARS-CoV PLpro may have deubiquinating activity on the basis of shared structural features with a cellular deubiquitinating enzyme, herpesvirus-associated ubiquitin-specific protease (HAUSP) (21, 53). Furthermore, SARS-CoV PLpro was predicted to cleave the consensus sequence LXG (54), which is also recognized by deubiquitinating enzymes. Sulea predicted that a “structural signature” in the substrate-binding pocket, which may be important for cleavage after the diglycine residues, is present in some, but not all, coronavirus papain-like proteases. Testing these intriguing predictions is critical for increasing our understanding of the role of proteases in coronavirus replication and for developing antiviral agents directed against this important target.

Here, we report the identification, purification, and characterization of a 316-amino-acid catalytic core domain of SARS-CoV PLpro that is soluble and highly active. Our studies provide experimental evidence supporting the hypothesis that PLpro requires a zinc-binding domain and a catalytic triad (Cys1651-His1812-Asp1826) instead of a catalytic dyad for protease activity. To measure the kinetics of protease and putative deubiquitinating activity, we purified a catalytic core of SARS-CoV PLpro and developed in vitro peptide hydrolysis and deubiquitinating assays. We found that purified PLpro hydrolyzed both diubiquitin and synthetic peptide substrates and that the ubiquitin substrate was hydrolyzed approximately 180-fold more efficiently than the peptide substrate. Finally, we show that SARS-CoV PLpro cleaves at the consensus cleavage site LXG, which is also the target sequence recognized by many deubiquitinating enzymes.

**MATERIALS AND METHODS**

**Cells.** HeLa-MHVR receptor cells were used for all transfection-based assays as previously described (25). The cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum, 2% penicillin-streptomycin, and 5 mM HEPES, pH 7.4.

**Construction of SARS-CoV PLpro expression plasmids and site-directed mutagenesis.** Regions of SARS-CoV PLpro were PCR amplified from parental plasmid pPLpro1541-2204 (17). The primer sequences are available on request. The PCR products were digested with the restriction enzymes EcoRI and BamHI, and the fragments were ligated into the corresponding sites of plasmid vector pcDNA3.1 V5His (Stratagene, La Jolla, Calif.). After transformation into Escherichia coli XL-1 Blue cells (Stratagene), bacteria were grown at 25°C.

Site-directed mutagenesis was performed to change specific residues in pPLPro1541-1855 or the substrate pNSPI-3* using synthetic oligonucleotides (sequences are available on request). QuikChange Mutagenesis (Stratagene) was performed according to the manufacturer’s instructions, and sequence changes were confirmed by DNA sequencing.

For purposes of overexpressing and purifying protein, DNA regions encoding core domains of wild-type and mutant PLpro enzymes were PCR amplified and cloned into pET11a (Invitrogen) between the NdeI and BpuI102I sites. The final clones were verified by DNA sequencing and designated pET-SARS-CoV-PLpro1541-1855 wild type, C1651A, and D1826A.

**Sequence comparison of coronavirus papain-like protease domains.** The amino acid sequence of the single papain-like protease found in aIBV and paragonav papain-like protease domains 1 and 2 found in seven other coronaviruses were aligned using the program ALIGN (SeeED) with the SARS-CoV PLpro catalytic core domain (amino acid sequences 1541 to 1855) as the reference sequence. The scoring matrix BLOSUM 62 was used to set the alignment parameters, and the similarity significance value cutoff was set at greater than or equal to 60%.

**Purification of SARS-CoV PLpro.** Three liters of E. coli BL21(DE3) cells, containing pET11a-SARS-CoV-PLpro1541-1855, were grown for 24 h at 25°C. Cells were pelleted by centrifugation and resuspended in 80 ml of buffer A (20 mM Tris, pH 7.5, 10 mM 2-mercaptoethanol [BME]) containing 500 µg of lysozyme. The cells were incubated for 10 min and then lysed via sonication using a 600-watt model VCVX ultrasonicator. After the cell debris was pelleted by centrifugation (40,000 × g for 30 min), the clarified cell lysate was subjected to a 40% ammonium sulfate fractionation. The suspension was centrifuged (40,900 × g for 30 min), and the resulting pellet was resuspended in 1 M ammonium sulfate. The suspension was centrifuged at 77,000 × g for 30 min, and the resulting pellet was resuspended in 1 M ammonium sulfate, 100 mM Tris, pH 7.5, 10 mM BME. The dissolved pellet was loaded on to a 50-mL phenyl-Sepharose 6 Fast-Flow HS column (Amersham BioSciences, Piscataway, NJ) equilibrated with buffer C (1.5 M ammonium sulfate, 20 mM Tris, pH 7.5, and 10 mM BME). Protein was eluted with a 10-column-volume gradient to 100% buffer A. Fractions containing SARS-CoV PLpro were pooled and diluted fivefold with buffer A and loaded onto a 50-mL Q-Sepharose Fast-Flow column (Amersham BioSciences, Piscataway, NJ) equilibrated with the same buffer. A 10-column-volume gradient to 100% buffer B (0.5 M NaCl, 20 mM Tris, pH 7.5, 10 mM BME) was used to elute the protein. SARS-CoV PLpro fractions were pooled, diluted fivefold with buffer A, and loaded onto a Mono Q 10/10 column (Amersham BioSciences, Piscataway, NJ). PLpro eluted under the same conditions as the Q-Sepharose column. Pure SARS-CoV PLpro fractions were exchanged into buffer A containing 20% glycerol, concentrated to approximatively 20 mg/ml, flash-frozen in dry ice-ethanol, and stored at −80°C. Site-directed mutants of SARS-CoV PLpro enzymes were expressed, purified, and stored in the same manner.

**Transfection and immunoprecipitation of SARS-CoV PLpro constructs.** Expression of SARS-CoV PLpro constructs was performed as previously described (17). Briefly, HeLa-MHVR cells were infected with recombinant vaccinia virus fT7 expressing T7 polymerase and transfected with plasmid DNAs pSARS-CoV-PLpro and/or pNSPI-3* substrate using Lipofectamine (Invitrogen). Proteins were metabolically labeled using 100 µCi/ml Trans-35S label for 4 h at 37°C. Cells were harvested by scraping into 0.3 ml of lysis buffer A (4% sodium dodecyl sulfate [SDS], 3% dithiothreitol, 40% glycerol, 0.065 M Tris, pH 6.8). Cell lysates were diluted in 1 ml of radioimmunoprecipitation assay buffer and immunoprecipitated with specific antibodies and protein A-Sepharose beads (Amersham Bioscience, Piscataway, N.J.). The following antibodies were used: rabbit polyclonal antibody anti-R1 to detect SARS-CoV nsp1 and anti-R3 to detect SARS-CoV nsp3, including SARS-CoV PLpro (17). Mouse monoclonal anti-V5 (Invitrogen) was used to detect epitope-tagged constructs as indicated. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

**Fluorescence and FRET assays for enzyme kinetics.** All assays were performed in 50 mM HEPES, pH 7.5, in a 500-µl cuvette maintained at 25°C using a Peltier sample changer. The rates of the reaction were monitored using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Palo Alto, CA). The fluorescence extinction coefficients of both fluorescent substrates were determined using the endpoint method as follows: an excess of SARS-CoV PLpro enzyme was added to various concentrations of substrate, and reactions were allowed to go to
RESULTS

Identifying a catalytic core domain of SARS-CoV PLpro. To identify a catalytic core domain of SARS-CoV PLpro, we performed sequential deletion analysis on pPLpro1541-2294, which we had previously shown to express an active protease in trans-cleavage assays (17). Initially, we deleted 280 amino acids from the C-terminal end of the construct to generate pPLpro1541-1924 (Fig. 1A). This construct was used as the parental construct for subsequent deletions of 20 to 30 amino acids from either the N- or C-terminal end of the PLpro domain. We tested each construct for expression of PLpro (Fig. 1B) and for protease activity in the trans-cleavage assay (Fig. 1C). We found that each construct expressed a PLpro domain of the expected size, and similar amounts of the PLpro product were immunoprecipitated by polyclonal antiserum that recognizes the SARS-CoV PLpro domain (Fig. 1B, lanes 1 to 10). When these constructs were cotransfected with plasmid DNA expressing the substrate NSP1-3*, we found that only 5 of the 10 expressed PLpro proteins were enzymatically active and able to cleave the substrate to produce the nsp1 product (Fig. 1C, lanes 1 to 3 and 6 to 7). Of these five functional PLpro domains, we found that the three C-terminal deletion constructs displayed more robust protease activity compared to the two N-terminal deletion products, as determined by monitoring the reduction of the NSP1-3* substrate. To determine if both C- and N-terminal regions could be truncated, we generated the double-deletion construct pPLpro1602-1855. However, this PLpro domain was no longer active in the trans-cleavage assay.

FIG. 1. Defining a core SARS-CoV PLpro domain using a trans-cleavage assay. (A) Schematic diagram of constructs generated to identify an active core domain of SARS-CoV PLpro. Starting with the fragment from residues 1541 to 1924, successive deletions of 20 amino acids were made from the C-terminal and N-terminal ends. The catalytic cysteine (C1651) and histidine (H1812) residues are indicated (17, 54). The constructs are designated by the residues at which they begin and end. (B) Expression of SARS-CoV PLpro deletion constructs. SARS-CoV PLpro deletion constructs were expressed via vaccinia-mediated T7 expression in HeLa cells. The proteins were radiolabeled with Tras35S, immunoprecipitated with antibody anti-R3, and resolved by SDS-PAGE. (C) trans-cleavage assay to assess PLpro activity. SARS-CoV PLpro deletion mutants were coexpressed with substrate NSP1-3* as shown, via vaccinia-mediated T7 expression in HeLa cells. The proteins were radiolabeled with Trans35S, immunoprecipitated with antibody anti-R1, which precipitates the uncleaved precursor NSP1-3* and cleavage product nsp1. Products were analyzed by electrophoresis on SDS–10% polyacrylamide gels and visualized by autoradiography. ORF, open reading frame.
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**Catalytic Cys**

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**Zinc-Binding Cys**

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TGEV, HCoV-NL63, HCoV-229E, and HCoV-OC43) encode two papain-like protease domains, termed either PLP1 and PLP2, or PLpro and PL2pro. Two coronaviruses, SARS-CoV and aIBV, encode only one papain-like protease domain, which we term PLpro. For our analysis, we selected the amino acid sequences of each virus that would be analogous to the SARS-CoV PLpro minimal domain (a 316-amino-acid region, with 110 amino acids upstream of the putative catalytic cysteine residue and 206 amino acids downstream). The multiple sequence alignment revealed that although the overall identity of amino acids in the coronavirus papain-like protease domain is low (18 to 32%), the amino acids likely to participate in the catalytic core are highly conserved (Fig. 2, boxed sequences). Previous experimental evidence and computer modeling suggested that coronavirus papain-like proteases possess a Zn\(^{2+}\) binding domain that connects the left- and right-hand domains of a papain-like fold and that protease activity is mediated by a Cys-His catalytic dyad (19, 26, 36). Interestingly, both our analysis and the recent modeling of Sulea and coworkers (53) identify an additional and conserved residue, D1826, which may also be important for PLpro activity (Fig. 2, boxed in red). We hypothesize that coronavirus papain-like proteases, like cellular papain-like proteinases, may employ a Cys-His-Asp catalytic triad that is assisted by a conserved residue (usually Q/N) occupying the oxyanion hole (6, 23, 37, 51). To determine if D1826 and the putative zinc-binding residues are important for protease activity, we performed site-directed mutagenesis and changed the nucleotide sequence to code for alanine at the selected sites. The resulting PLpro proteins were tested for activity in the \(\text{trans}\)-cleavage assay (Fig. 3). As predicted, we found that alanine substitution of the conserved residues (C1651, D1826, and putative zinc-binding residues C1729, C1732, C1764, and C1766) resulted in the complete loss of PLpro activity as observed via \(\text{trans}\)-cleavage assays. In contrast, alanine substitution of a nonconserved cysteine (C1688) residue did not affect protease activity. Overall, these results support the hypothesis that the zinc-binding domain is important for the maintenance of the active site and that coronavirus papain-like proteases may exploit a Cys-His-Asp catalytic triad similar to cellular papain-like proteases.

Sulea and coworkers also predicted that a tryptophan residue in position 1646 may serve as a putative oxyanion-hole residue for SARS-CoV PLpro. The oxyanion-hole is usually filled by a glutamine or asparagine residue, and we hypothesize that the highly conserved glutamine residue downstream from the catalytic cysteine (residue 1661 in SARS-CoV PLpro) may function as an oxyanion-hole residue (Fig. 2, boxed in red). However, we found that alanine substitution of Q1661 did not abolish SARS-CoV PLpro activity in the \(\text{trans}\)-cleavage assay, suggesting that this residue does not play a critical role in processing (data not shown). Ultimately, structural data will be essential for defining the SARS-CoV PLpro active site.

Our bioinformatic analysis was also consistent with the prediction of Sulea et al. (53) that suggested coronavirus PLpro domains have a signature, substrate-binding site that may support deubiquitinating activity. By mining the current Protein Data Bank, Sulea and coworkers predicted that the SARS-CoV PLpro sequence from K1632 to E1847 may have a structure similar to the catalytic core domain of the cellular protein USP7, which is also known as HAUSP. This cysteine protease has been shown to have deubiquitinating activity (21). Molecular modeling of SARS-CoV PLpro suggests that this protease, like HAUSP and other deubiquitinating enzymes (DUBs), may recognize substrates with the sequence LXGG. Sulea and coworkers noted that the model predicts that significant occlusions of the S₁ and S₂ subsites account for the strict specificity for the predicted diglycine substrate. Furthermore, they noted that these “structural signatures for strict specificity,” which we term the “DUB signature sequences,” were present in HCoV-229E PLpro and PL2pro and MHV PLP2, but not MHV PLP1. Our analysis extends these observations, and we noted that 12 of the 16 coronavirus papain-like protease domains retain the conserved signature sequence (Fig. 2, boxed in blue). Only four PLpro domains (HCoV-HKU1 PL1, MHV PLP1, HCoV-OC43 PL1P1, and BCoV PLP1) do not retain the signature sequence and therefore would not be predicted to exhibit deubiquitinating activity. To determine if the putative “DUB signature residues” are essential for SARS-CoV PLpro activity, we generated alanine substitution mutants and tested them for activity in the \(\text{trans}\)-cleavage assay (Fig. 3, lanes 10 and 11). As predicted, replacement of the Y1804 or Y1813 resulted in a loss of protease activity. These results provide the first experimental evidence that two of the residues in the DUB signature sequences are important for maintaining the active site.

**Purification of SARS-CoV PLpro.** Ultimately, our goal is to purify an active form of SARS-CoV PLpro for use in a high-throughput assay to screen for inhibitors that block protease activity, as well as for crystallization and X-ray structure determination. As a first step toward these goals, we expressed SARS-CoV PLpro1541-1855 in *E. coli* BL21(DE3) cells, purified the protein to homogeneity, and developed a FRET-based peptide cleavage assay to measure the kinetics of SARS-CoV PLpro activity. The purification consisted of a combination of hydrophobic interaction and anion exchange chromatography and resulted in the purification of a protein that resolved as a...
the highest concentration of peptide tested, i.e., 400 µM (data not shown). Based on the observation that enzyme activity was linearly proportional to increasing concentration of the peptide substrate (Fig. 4B), the initial velocity measurements were plotted against substrate concentration to determine the pseudo first-order rate constant, $k_{\text{app}}$, for SARS-CoV PLpro activity (Fig. 4B) ($k_{\text{app}}$ approximates $k_{\text{cat}}/K_m$ for nonsaturable enzymes). The pseudo first-order rate constant for wild-type SARS-CoV PLpro, calculated from rates obtained at different peptide concentrations was determined to be $(2.44 \pm 0.03) \times 10^{-2} \text{ min}^{-1} \text{ M}^{-1}$ (Table 1). The SARS-CoV PLpro active-site mutant, C1651A, was completely inactive with the peptide substrate. However, SARS-CoV PLproD1826A, a putative catalytic triad mutant, was determined to be slightly active with approximately a 100-fold decrease in the $k_{\text{app}}$ value, which is $(2.3 \pm 0.2) \times 10^{-4} \text{ min}^{-1} \text{ M}^{-1}$. These results are consistent with studies of other papain-like proteases and deubiquitinating enzymes, where it was shown that the cysteine residue plays an essential and critical role in the nucleophilic attack of the reaction and that the aspartic acid plays a significant, although not essential role, in orienting and/or stabilizing the substrate in the active site (23, 31).

Purified SARS-CoV PLpro was also tested for deubiquitinating activity in vitro using a commercially available fluorescent substrate, ubiquitin-AMC. As with the fluorescent peptide, saturation could not be achieved with the ubiquitin substrate at the concentrations assayed. Interestingly, SARS-CoV PLpro showed a strong preference for the ubiquitin substrate over the peptide substrate with a deubiquitinating activity that is 180-fold more efficient ($k_{\text{app}}$ value of 4.48 ± 0.11 min$^{-1}$ µM$^{-1}$) than with the peptide substrate (Fig. 4B and Table 1). Indeed, SARS-CoV PLpro is considerably more active against the ubiquitin-AMC substrate than HAUSP ($k_{\text{cat}}/K_m = 13 \text{ min}^{-1} \text{ mM}^{-1}$[21]). For both SARS-CoV PLpro and HAUSP enzymes, $K_m$ values could not be determined, since saturation of the enzymes with peptide substrates could not be achieved (21). Therefore the $k_{\text{app}}$ for SARS-CoV PLpro and the reported $k_{\text{cat}}/K_m$ for HAUSP indicate that these enzymes have low affinities for the peptide substrates tested but substantial turnover rates. In contrast, the $K_m$ values observed for several other ubiquitin C-terminal hydrolases family members are in the range of 1 to 3 µM, indicating that they have higher affinity for their substrates (10, 24, 30, 32). SARS-CoV PLproC1651A and PLproD1826A were also tested for deubiquitinating activity; PLproC1651A was again completely inactive, whereas PLproD1826A displayed only 1% the activity of

![Image](http://jvi.asm.org/)

**FIG. 3.** Identifying residues essential for proteolytic activity of SARS-CoV PLpro. SARS-CoV PLpro constructs encoding the wild-type sequence or specific alanine substitutions were coexpressed with substrate NSP1-3* as described in the legend of Fig. 1C to identify residues essential for SARS-CoV PLpro activity. The site of the alanine substitution is indicated above each lane. WT, wild type.
wild-type enzyme at the 0.5 μM concentration of ubiquitin-
AMC substrate (data not shown).

To confirm these findings using an independent assay, we
tested purified SARS-CoV PLpro wild-type and the C1651A
and D1826A mutant enzymes for their ability to cleave a di-
ubiquitin substrate (21). SARS-CoV PLpro enzymes were in-
cubated with the diubiquitin substrate for 5, 15, and 60 min,
and the products of the reaction were resolved by SDS-PAGE
and visualized by staining with Coomassie blue (Fig. 4C). The
diubiquitin substrate was efficiently cleaved by wild-type
SARS-CoV PLpro, demonstrating its DUB activity. The cata-
lytic cysteine mutant, PLproC1651A, was completely inactive
in this assay, and PLproD1826A had a reduced but detectable
level of enzymatic activity. Thus, both fluorescence-based and
protein-based assays provided experimental evidence for
SARS-CoV PLpro DUB activity.

SARS-CoV PLpro recognizes and cleaves at the LXGG con-
sensus cleavage site. Previous studies have predicted and pro-
vided some experimental evidence that SARS-CoV PLpro rec-
ocognizes the consensus sequence LXGG at the three cleavage
sites in the replicase polyprotein (16, 17, 54). Interestingly, this
cleavage site is also the consensus cleavage site recognized by
DUB enzymes such as HAUSP (21, 53). To determine if the
predicted LXGG consensus site is indeed important for PLpro
recognition and processing, we confirmed the location of each
of the three cleavage sites and performed site-directed mu-
hypothesis that SARS-CoV PLpro exploits a catalytic triad to recognize and process ubiquitinated substrates. Using bioinformatic analysis, we identified conserved residues within the PLpro domain of 316 amino acids that can process the amino-protease with DUB activity. We identified a core SARS-CoV PLpro recognition and processing at each cleavage site. The consensus LXGG sequence is important for SARS-CoV PLpro recognition and processing at these two sites.

In this study, we show that SARS-CoV PLpro is a viral protease with DUB activity. We identified a core SARS-CoV PLpro domain of 316 amino acids that can process the aminoterminal portion of the SARS-CoV replicase polyprotein and can recognize and process ubiquitinated substrates. Using bioinformatic analysis, we identified conserved residues within this core and provided experimental evidence to support the hypothesis that SARS-CoV PLpro exploits a catalytic triad to mediate proteolytic processing and DUB activity. Furthermore, SARS-CoV PLpro recognizes and cleaves substrates with the consensus sequence LXGG, which is also the consensus sequence processed by many cellular DUBs. These findings have important implications for the development of antiviral agents to PLpro and open new avenues of investigation concerning the role of a viral DUB activity in coronavirus replication and pathogenesis.

Current antiviral drug development is driven by two major approaches: high-throughput screening (HTS) of chemical compound libraries and rational drug design based on structural information. The identification of the core domain of PLpro and the development of in vitro assays for enzymatic activity described here will facilitate HTS for SARS-CoV PLpro inhibitors. The purified SARS-CoV PLpro core domain retained enzymatic activity in vitro, and kinetic studies indicated deubiquitinating activity was even more robust than hydrolysis of the 12-mer peptide substrate. It may be challenging to develop PLpro inhibitors that are specific for the viral protease, without blocking cellular DUBs. However, the structural differences in cellular DUBs suggest that these enzymes may be different enough to be selectively targeted. Further information from structural studies will be required to better understand and exploit any potential differences. We note that HTS using the other SARS-CoV protease, 3CLpro, has already led to the identification of many small-molecule inhibitors (7). In addition, HTS against SARS-CoV-infected cells has lead to the identification of small molecules that target 3CLpro and block SARS CoV replication (59). The former approach could also be used to develop HTS assays for papain-like protease domains from other human coronaviruses such as the recently identified NL-63 (12, 55) and HKU-1 (58) and “common cold” coronaviruses OC43 and 229E (20).

<table>
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<th>SARS-CoV PLpro</th>
<th>Deubiquitination $k_{app}$ (min $^{-1}$uM $^{-1}$)</th>
<th>Peptide hydrolysis $k_{app}$ (min $^{-1}$uM $^{-1}$)</th>
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<td>$(2.44 \pm 0.03) \times 10^{-2}$</td>
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<tr>
<td>C1651A</td>
<td>No activity</td>
<td>No activity</td>
</tr>
<tr>
<td>D1826A</td>
<td>$&lt;1%$ activity of WT</td>
<td>$(2.3 \pm 0.2) \times 10^{-4}$</td>
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$^a$ WT, wild type.

$^b$ Substrate for deubiquitination: ubiquitin-AMC (cleavage site: LRGG–AMC). The PLpro recognition site is underlined, and the cleavage site is indicated by a `_`.

$^c$ Substrate for peptide hydrolysis: (E-EDANS)RELNGG–API(K-DABCYL). See footnote $b$ for explanation of underlining and `_`.

FIG. 5. SARS-CoV PLpro recognition and processing of LXGG consensus substrate cleavage sites. Alanine (or asparagine, indicated by #) substitutions were made at the P6 to P2* positions of the three SARS-CoV PLpro substrates: API(K-DABCYL) –cleavage site: LRGG–AMC. The PLpro recognition site is underlined, and the cleavage site is indicated by a `_`.

Current antiviral drug development is driven by two major approaches: high-throughput screening (HTS) of chemical compound libraries and rational drug design based on structural information. The identification of the core domain of PLpro and the development of in vitro assays for enzymatic activity described here will facilitate HTS for SARS-CoV PLpro inhibitors. The purified SARS-CoV PLpro core domain retained enzymatic activity in vitro, and kinetic studies indicated deubiquitinating activity was even more robust than hydrolysis of the 12-mer peptide substrate. It may be challenging to develop PLpro inhibitors that are specific for the viral protease, without blocking cellular DUBs. However, the structural differences in cellular DUBs suggest that these enzymes may be different enough to be selectively targeted. Further information from structural studies will be required to better understand and exploit any potential differences. We note that HTS using the other SARS-CoV protease, 3CLpro, has already led to the identification of many small-molecule inhibitors (7). In addition, HTS against SARS-CoV-infected cells has lead to the identification of small molecules that target 3CLpro and block SARS CoV replication (59). The former approach could also be used to develop HTS assays for papain-like protease domains from other human coronaviruses such as the recently identified NL-63 (12, 55) and HKU-1 (58) and “common cold” coronaviruses OC43 and 229E (20).
the purification of PLpro is a critical first step toward obtaining structural information on this important therapeutic target. This study also raises important questions concerning the role of a viral DUB activity in coronavirus replication and pathogenesis. Cellular DUBs are known to alter the fate of proteins targeted to specific pathways in the cell (1). Deubiquitination may change the fate of (i) ubiquitinated proteins bound for degradation in the proteasome (56), (ii) proteins modified by ubiquitin-like (UBL) modifications such as ISGylation (46), and (iii) proteins assembled in autophagosomes that are bound for degradation in lysosomes (48). Modulation of any of these pathways may have important implications on viral replication and pathogenesis. For example, HAUSP, the cellular DUB activated by herpesvirus infection, plays a dynamic role in the stabilization of p53. HAUSP can act directly to deubiquitinate p53, thereby stabilizing its tumor suppressor function (34). In addition, HAUSP acts on Mdm2, the ubiquitin ligase required to modify p53. In the absence of HAUSP, Mdm2 auto-ubiquitinates and is degraded, and p53 is stable in the cell (9, 33). This illustrates the dynamic effect that DUBs can have on protein stabilization. It is currently unclear if SARS-CoV PLpro specifically targets and stabilizes a cellular substrate. Further investigation is necessary to determine if specific substrates are recognized and stabilized by PLpro.

One interesting question is whether the SARS-CoV PLpro peptide hydrolysis and DUB activities can be separated. A better understanding of enzyme-substrate interactions is needed to identify distinct contact residues that may play a role in distinguishing one substrate from another.

Sulea et al. speculated that SARS PLpro DUB activity may function to inhibit innate immune responses such as conjugation with ISG15 [interferon (IFN)-sensitive gene 15], termed ISGylation (44, 53). ISG15, a ubiquitin-like protein, is induced in response to IFN-α/β. While the role of ISG15 is not entirely clear, it is conjugated to many targets, including JAK and STAT proteins, and this conjugation leads to suppression of cell proliferation (36). The adenovirus protease has been shown to have both deubiquitinating and “de-ISGylating” activity and recognizes the LXXG consensus site for removal of ISG15 (5). In addition, the influenza virus NS1 protein has been shown to inhibit the conjugation of ISG15, thereby counteracting this IFN-induced activity (62). Interestingly, recent studies indicate that SARS-CoV infection may escape IFN-mediated growth inhibition by blocking the activation of IFN regulatory factor 3 (49). The mechanisms and viral proteins required for this IFN-antagonistic effect are currently unclear. Experiments are currently under way to determine if ISG15 is activated and if levels of ISGylated proteins are altered in coronavirus-infected cells.

Another intriguing speculation is that SARS-CoV PLpro activity may be involved in subverting a normal cellular process known as autophagy to facilitate the assembly of DMVs, which are the site of coronavirus replication (14, 43). Autophagy is a cellular response to starvation conditions, whereby the cell recycles cytoplasmic components by enclosing them in double-membrane structures known as autophagosomes for delivery to lysosomes for degradation (48). At least 16 genes (termed Apg for autophagy-defective) and two UBL modification systems are required for autophagy in *Saccharomyces cerevisiae* (38, 39). In the first UBL system, Apg12 is activated by Apg7 and Apg10, which are E1-like and E2-like enzymes, respectively, of the ubiquitin system. Apg12 is then conjugated to Apg5, which is required for assembly of autophagosomes and viral DMVs (43). In the second UBL system, Apg8 is activated by E1-like and E2-like enzymes, Apg7 and Apg3, and is conjugated with phosphatidylethanolamine, making it a membrane-associated complex. Studies of both coronavirus and poliovirus replication complexes indicate that viral replicase proteins may assemble with intracellular membranes to form DMVs, which are the site of viral replication (13, 14, 40, 43, 52). The mechanism for the assembly of the DMVs is not clear, but recent studies suggest that viral proteins may subvert the cellular autophagosomal machinery (22, 28, 43). For example, LC3, the mammalian homolog of yeast Atg8, has been implicated in the assembly of viral DMVs since it colocalizes with coronavirus and poliovirus replication complexes (22, 43). We speculate that coronaviruses may exploit the cellular autophagy pathway for the assembly of the DMVs but then subvert the pathway by deubiquitination to prevent the maturation of the vesicles into degradable organelles. Experiments are currently in progress to test these hypotheses.

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