Structurally Guided Removal of deISGylase Biochemical Activity from Papain-Like Protease Originating from Middle East Respiratory Syndrome Coronavirus

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ABSTRACT Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging human pathogen that is the causative agent for Middle East respiratory syndrome (MERS). With MERS outbreaks resulting in over 35% fatalities and now spread to 27 countries, MERS-CoV poses a significant ongoing threat to global human health. As part of its viral genome, MERS-CoV encodes a papain-like protease (PLpro) that has been observed to act as a deubiquitinase and deISGylase to antagonize type I interferon (IFN-I) immune pathways. This activity is in addition to its viral polypeptide cleavage function. Although the overall impact of MERS-CoV PLpro function is observed to be essential, difficulty has been encountered in delineating the importance of its separate functions, particularly its deISGylase activity. As a result, the interface of MERS-CoV and human interferon-stimulated gene product 15 (hISG15) was probed with isothermal calorimetry, which suggests that the C-terminal domain of hISG15 is principally responsible for interactions. Subsequently, the structure of MERS-CoV PLpro was solved to 2.4 Å in complex with the C-terminal domain of hISG15. Utilizing this structural information, mutants were generated that lacked appreciable deISGylase activity but retained wild-type deubiquitinase and peptide cleavage activities. Hence, this provides a new platform for understanding viral deISGylase activity within MERS-CoV and other CoVs.

IMPORTANCE Coronaviruses, such as Middle East respiratory syndrome coronavirus (MERS-CoV), encode a papain-like protease (PLpro) that possesses the ability to antagonize interferon immune pathways through the removal of ubiquitin and interferon-stimulated gene product 15 (ISG15) from target proteins. The lack of CoV proteases with attenuated deISGylase activity has been a key obstacle in delineating the impact between deubiquitinase and deISGylase activities on viral host evasion and pathogenesis. Here, biophysical techniques revealed that MERS-CoV PLpro chiefly engages human ISG15 through its C-terminal domain. The first structure of MERS-CoV PLpro in complex with this domain exposed the interface between these two entities. Employing these structural insights, mutations were employed to selectively remove deISGylase activity with no appreciable impact on its other deubiquitinase and peptide cleavage biochemical properties. Excitingly, this study introduces a new tool to probe the pathogenesis of MERS-CoV and related viruses through the removal of viral deISGylase activity.

KEYWORDS coronavirus, ISG15, Middle East respiratory syndrome, papain-like protease

Middle East respiratory syndrome (MERS) is a devastating human disease involving more than 1,700 cases spanning 27 different countries, with an overall case fatality rate of just over 35% (1). The causative agent for MERS, MERS coronavirus (MERS-CoV), is an enveloped, positive-sense single-stranded RNA (ssRNA) virus classified as belonging to the *Betacoronavirus* genus of the *Coronaviridae* family. MERS-CoV

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was first isolated in 2012 from a 60-year-old man originating from Saudi Arabia who suffered from respiratory symptoms and later died from severe progressive respiratory and renal failure (2). Shortly thereafter, additional MERS-CoV cases were observed originating from infected dromedary camels as well as human-to-human transmission (3, 4). Due to the resemblance and severity in the symptoms brought on by infection, proven human-to-human transmission, and its zoonotic nature, MERS-CoV is often compared to severe acute respiratory syndrome coronavirus (SARS-CoV) (4). With SARS-CoV causing a pandemic, with more than 8,000 cases (~10% case fatality rate) in 2003, there is considerable concern about a potential, similar MERS-CoV outbreak (5, 6). As a result, MERS-CoV is currently seen as an emerging global human health threat.

As with other positive-stranded RNA viruses, coronaviruses encode long viral polyproteins containing 16 different nonstructural proteins (Nsp1 to Nsp16) that are cleaved by virus-encoded proteases (7–9). For coronaviruses in general, they encode one 3C-like protease, also known as the main protease, as well as up to two papain-like proteases (PLPs). In the case of MERS-CoV, only one PLP is encoded, papain-like protease (PLpro). The MERS-CoV PLpro and 3C-like protease are responsible for cleaving Nsp1 to Nsp3 and Nsp4 to Nsp16, respectively, in order to generate the membrane-bound replicase complex necessary for RNA replication (8, 10). Intriguingly, CoV PLpros have been observed to have additional roles beyond simply cleaving Nsp1 to Nsp3 to promote viral replication (11).

Similar to SARS-CoV PLpro and some other CoV PLPs, MERS-CoV PLpro possesses the ability to reverse posttranslational modifications by ubiquitin (Ub) and interferon-stimulated gene product 15 (ISG15) (Fig. 1) (12). Ubiquitination and ISGylation of viral and host proteins have been observed to play essential roles in several immune pathways to include the type I interferon (IFN-I), which is often viewed as the first line of defense against viral infection (13, 14). This includes the production and release of IFNs within the IFN-I response as well as the NF- κ B inflammation response (15). Additionally, these posttranslational modifications are central to the increased production of chemokines and cytokines as well as other IFN-stimulated gene products with antipathogenic properties (15, 16). In line with the importance of ubiquitination and ISGylation in host immunity, the dual deubiquitinase and deISGylase activities of the SARS-CoV PLpro were observed to antagonize the IFN-I response and were suggested to be a key viral evasion mechanism (15, 16). This was further supported by the diminishment of deubiquitinase and deISGylase activities in the mouse hepatitis coronavirus (MHV) PLpro equivalent through use of a destabilizing mutation (10). Also, direct disruption of MERS-CoV PLpro's deubiquitinase and deISGylase proteolytic activities together was shown to remove antagonism of the IFN-I response in part due to suppression of Ub-dependent mitochondrial antiviral signaling protein (MAVS)-mediated signaling (17).

Regrettably, as pointed out by the authors in the latter study, a lack of structural information pertaining to how MERS-CoV PLpro accommodates ISG15s limited their ability to delineate whether the MERS-CoV PLpro's deubiquitinase, deISGylase, or both activities were critical for effective IFN-I antagonism by MERS-CoV or other CoV PLPs (17). To date, several structures of MERS-CoV PLpro bound to Ub have been solved, illuminating the specific amino acid residues involved in ubiquitin binding (17, 18). However, no structures illuminating the interface of MERS-CoV and ISG15s have similarly been elucidated. Some structural information on the interactions between a CoV PLpro and ISG15 has recently become available through the structures of SARS-CoV PLpro in complex with the C-terminal domain from two species of ISG15s (19). This study highlighted significant differences in how SARS-CoV PLpro accommodated not only ubiquitin differently than human ISG15 (ISG15) but also in how the accommodation of ISG15s from different species could differ. However, it also demonstrated that the thermodynamic factors governing MERS-CoV engagement of human ISG15 differ from those of SARS-CoV PLpro (Table 1) (19). Additionally, MERS-CoV preferred a different subset of ISG15 species, suggesting that mutations that may shift or limit

FIG 1 Sequence alignment of PLpros and PLP2s from coronaviruses. The PLpro or PLP2 from MERS-CoV (GenBank accession number [AF588944](#)), SARS-CoV (accession number [P0C6U8](#)), human coronavirus NL63 (HCoV-NL63) (accession number [YP_003766](#)), HCoV-OC43 (accession number [AMK59674](#)), HCoV-229E (accession number [APT69896](#)), and HCoV-HKU1 (accession number [ARB07606](#)). The residue numbering is based on MERS-CoV PLpro. The secondary structure predicted by the dictionary of protein secondary structure (DSSP) is shown for MERS-CoV PLpro. Residues that form the catalytic triad are boxed in red, while residues forming the zinc finger motif are boxed in purple. The residues mutated in this study are denoted by stars, which are colored based on the enzymatic activity results. Mutants shown in green have reduced activity toward ISG15-AMC; mutants shown in cyan have increased activity toward ISG15-AMC. The mutant shown in magenta showed little change in activity and the mutant shown in gray showed increased activity toward Ub-AMC. The mutants shown in purple represent the N-terminal mutants.

certain deubiquitinase and that species-specific delSGylase activities in SARS-CoV PLpro may not be directly translatable to MERS-CoV PLpro (19).

To address these issues, we determined through isothermal titration calorimetry (ITC) and mutational data that MERS-CoV PLpro principally engages human ISG15 through its C-terminal domain (ChISG15). Additionally, we solved the crystal structure of the MERS-CoV PLpro in complex with the C-terminal domain of human ISG15. Leveraging this structural information, amino acid sites within the MERS-CoV and hISG15 interface were probed to gain insight into the explicit interactions driving the ISG15–MERS-CoV PLpro binding event. This has resulted in the generation of altered MERS-CoV PLpros lacking appreciable biochemical delSGylase activity while still retaining wild-type (WT) deubiquitinase and peptide catalytic activities.

RESULTS AND DISCUSSION

Affinity of MERS-CoV PLpro for hISG15. MERS-CoV PLpro as well as other PLPs originating from CoVs has been widely observed to possess a dual role in promoting

TABLE 1 Isothermal titration calorimetry of human ISG15 and mono-Ub binding to CoV PLpros

Protein complex	No. of sites ^a	K_D (μ M)	ΔH (kJ/mol) ^b	ΔG (kJ/mol) ^c	$-T\Delta S$ (kJ/mol) ^d
MERS-CoV PLpro					
Human ISG15 ^e	0.785 $\pm$ 0.027	59.30 $\pm$ 12.7	−11.34 $\pm$ 0.268	−24.15	−12.80
C-human ISG15 ^f	0.721 $\pm$ 0.025	27.06 $\pm$ 3.04	−15.22 $\pm$ 0.521	−26.08	−10.87
Mono-Ub ^f	0.692 $\pm$ 0.011	9.56 $\pm$ 0.233	−21.20 $\pm$ 0.141	−6.85	14.30
SARS-CoV PLpro					
Human ISG15 ^e	0.932 $\pm$ 0.032	20.50 $\pm$ 4.48	−27.20 $\pm$ 1.90	−26.80	0.38
C-human ISG15 ^e	1.31 $\pm$ 0.015	57.60 $\pm$ 3.21	−4.03 $\pm$ 0.019	−24.18	−20.17
MERS-CoV PLpro K176E					
Human ISG15		>50 mM			
Mono-Ub ^f	0.655 $\pm$ 0.036	7.41 $\pm$ 0.643	−19.90 $\pm$ 0.00	−7.00	12.9
MERS-CoV PLpro V210D					
Human ISG15		>50 mM			
Mono-Ub ^f	0.708 $\pm$ 0.0041	200.50 $\pm$ 7.77	−16.00 $\pm$ 0.566	−5.045	10.95

^aBinding stoichiometry.^bBinding enthalpy.^cGibb's free energy.^dEntropy factor.^eData taken from Daczkowski et al. (19).^fAverage with error calculated using standard deviation. C-human ISG15, C-terminal domain of human ISG15.

viral replication through the processing of the viral polypeptide and the removal of posttranslational modifications of Ub and ISG15 (7–9, 11). For the former, the viral polypeptide cleavage activity appears to be largely dependent on the protease's ability to cleave the peptide bond after the LXGG recognition sequence found throughout the polypeptide (7, 9, 20). This differs for Ub and ISG15, where these proteases not only recognize a conserved LRLRGG sequence but also have been suggested to engage other tertiary elements of Ub and ISG15 (12, 17, 18). Recently, ITC data, as well as other mutational data, supported the idea that this interaction extends to the N-terminal domain of ISG15 for SARS-CoV PLpro (19, 20). Specifically, the C-terminal domain of hISG15 (ChISG15) was found to have weaker affinity and a different thermodynamic profile than full-length hISG15, supporting a role of the N-terminal domain in shaping the interactions between ISG15s and SARS-CoV PLpro (12, 19).

To assess whether the presence of the N-terminal domain of hISG15 had a similar influence on hISG15 binding to MERS-CoV PLpro, the affinity of MERS-CoV PLpro for ChISG15 was assessed utilizing ITC. The dissociation constant (K_D) for ChISG15 and MERS-CoV was 27.06 $\pm$ 3.04 μ M, which was weaker than that of mono-Ub (K_D = 9.56 $\pm$ 0.233 μ M), but ChISG15 showed slightly tighter binding than the previously published ITC data pertaining to the full-length PLpro (K_D = 59.3 $\pm$ 4.5 μ M) (Table 1 and Fig. 2). Surprisingly, the overall thermodynamics driving the interaction of the ChISG15 and the protease were strikingly similar to those of full-length ISG15. Specifically, like hISG15, the ChISG15 binding event was driven by a combination of favorable entropic and enthalpic components with similar ratios. This was orthogonal to the striking thermodynamic differences previously observed between SARS-CoV PLpro's interactions with hISG15 and ChISG15. Naturally, the full-length hISG15 could bind to MERS-CoV PLpro in a manner wherein the entropic and enthalpic contributions of the N terminus are masked by offsets in other interactions. However, a simpler explanation may be that the N-terminal domain of hISG15 does not play as significant a role for MERS-CoV PLpro as it does for SAR-CoV PLpro. This explanation would also imply that the C-terminal domain of hISG15 plays the dominant role in forming favorable interactions with MERS CoV PLpro.

Binding interface of MERS-CoV PLpro and ChISG15. To reveal key interactions of the C-terminal domain of hISG15 with MERS-CoV PLpro, the X-ray structure of the MERS-CoV PLpro-ChISG15 complex was initially determined to 2.7 Å in the P4₃ space group by collecting single-wavelength anomalous dispersion (SAD) data on the zinc

FIG 2 Isothermal titration calorimetry. (A) ITC binding isotherm with the raw heat (top panel) and integrated heats of injection (bottom panel) shown for the interaction between MERS-CoV PLpro and ChISG15. (B) ITC binding isotherm with the raw heat (top panel) and integrated heats of injection (bottom panel) shown for the interaction between MERS-CoV PLpro and MERS-CoV PLpro mutants, K176E and V210D, and mono-Ub. DP, differential heat power.

absorption edge. This structure was used as a search model to perform molecular replacement into a higher-resolution native data set. The subsequent 2.4-Å structure was found to possess two copies of the MERS-CoV PLpro-ChISG15 complex within the asymmetric unit (Table 2). Within each complex, densities for the protease's Ub-like domain and palm, thumb, and zinc-bound finger domains were present. In addition, the Ub-like fold of ChISG15 was found in close proximity to the fingers and palm domains of MERS-CoV PLpro (Fig. 3).

Examination of the MERS-CoV PLpro-ChISG15 binding interface revealed a mix of hydrophobic and electrostatic interactions that appears reflective of favorable entropic and enthalpic thermodynamic forces observed by ITC. Specifically, the MERS-CoV PLpro-ChISG15 structure revealed two sets of hydrophobic interactions. The most extensive of these hydrophobic interactions is generated by the accommodation of Leu85 and Pro144 along with the alkyl part of the Lys143 and Leu145 side chains of the ChISG15 interacting with a hydrophobic seam running along the fingers domain and Zn binding site of MERS-CoV PLpro. Specifically, this seam on the protease includes Val225 as well as the alkyl parts of the side chains belonging to Gln227 and Arg232 (Fig. 3B, frame 1). In addition, a smaller hydrophobic pocket, within the thumb and palm domains of the protease, is made up of interactions between His171 and Arg168 of the MERS-CoV PLpro and ChISG15 residues Pro130 and Trp123 (Fig. 3B, frame 2). Beyond hydrophobic interactions, two principle sets of electrostatic interactions are observed. One of these interactions is made via H bonds between Glu132 and Lys176 of the ChISG15 and MERS-CoV PLpro, respectively (Fig. 3B, frame 3). The other is comprised of the insertion of ChISG15's Lys129 side chain into a pocket generated by the carbonyl groups of the PLpro's Lys205 and Cys208 (Fig. 3B, frame 4). Overall, the mix of interactions appears to mirror the favorable entropic and enthalpic thermodynamic forces observed when the ISG15 product binds to the protease via ITC.

Differences in the accommodation of ChISG15 and Ub by CoV PLpros. Comparison of the MERS-CoV PLpro's accommodation of ChISG15 within its Ub binding motif (UIM) to that of Ub highlights the likely dynamic nature of PLpros' engagement of their substrates. Previously, MERS-CoV PLpro has been observed to adopt at least two states upon binding Ub in what has been proposed as a closed state and an open state (17). Alignment of the MERS-CoV PLpro bound to ChISG15 with these Ub-bound structures reveals that the protease in the MERS-CoV PLpro-ChISG15 adopts a conformation mirroring that of a closed state (Fig. 4A) (17). More explicitly, the Zn finger

TABLE 2 Data collection and refinement statistics for MERS-CoV PLpro-ChISG15

Parameter	Value for the parameter ^a	
	Zn-SAD (PDB 5W8T)	Native (PDB 5W8U)
Data collection		
Space group	P4 ₃	P4 ₃
Wavelength (Å)	1.2837	1
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	86.3, 86.3, 223.6	86.4, 86.4, 224.1
α , β , γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	50.00–2.76 (2.81–2.76)	50.00–2.41 (2.45–2.41)
<i>R</i> _{merge}	0.090 (0.922)	0.062 (0.766)
CC _{1/2} ^b	0.999 (0.838)	0.998 (0.728)
<i>I</i> / σ <i>I</i>	24.1 (1.5)	31.6 (1.49)
Completeness (%)	99.73 (99.59)	99.45 (98.59)
Redundancy	8.5 (7.8)	4.9 (3.5)
Phasing statistics		
No. of Zn sites found	7	
Phasing figure of merit	0.279	
Refinement		
Resolution (Å)	41.23–2.76 (2.86–2.76)	38.63–2.41 (2.45–2.41)
No. of reflections	41,870	62,588
<i>R</i> _{work} / <i>R</i> _{free} (%)	18.8/22.4	21.3/23.7
No. of atoms		
Protein	6,200	6,216
Ligand/ion ^c	62	78
Water	31	161
B factor		
Protein	84.42	67.43
Ligand/ion ^c	94.42	68.37
Water	62.77	63.37
RMS deviations ^d		
Bond length (Å)	0.006	0.004
Bond angle (°)	0.80	0.62

^aValues in parentheses denote the highest resolution shell.^bPearson correlation coefficient between to random half-data sets.^cIncludes the propargyl linker, MPD from the crystallization condition, and Zn.^dRMS, root mean square.

domain found in the MERS-CoV PLpro-ChISG15 structure forms additional interactions by wrapping around the ChISG15. This interaction is similar to that found in the previously described closed state of MERS-CoV proteases bound to Ub (Protein Data Bank [PDB] accession number [4FR0](#)) (17). This differs from the open state of the protease (PDB [4RF1](#)) where the Zn fingers are found peeled away from Ub, limiting the protease's interaction with the substrate (Fig. 4A).

Although MERS-CoV PLpro-ChISG15 resembles the closed state of MERS-CoV PLpro-Ub, the Ub molecule is oriented slightly differently within the UIM pocket relative to ChISG15. Closer examination of the active site illustrates that this deviation from the backbone perspective begins after Leu154 of ISG15 and increases toward the Ub fold motif of ChISG15 (Fig. 4B). In large part, the shared residues of the RLRGG C-terminal tail of Ub and ISG15 engage the protease in a similar manner. The lone exception is ISG15's Arg155, whose long flexible side chain forms hydrostatic interactions with the carbonyl of Val276 of the protease instead of Pro163, as seen with Ub's Arg74. However, this change appears to have minimal impact on the backbone, suggesting that the differences in orientation of ISG15 versus Ub within the UIM are likely the primary driver of the backbone deviations between the ISG15 and Ub residues within the active site. Given that the *k*_{cat} values of MERS-CoV PLpro for ISG15 and Ub are comparable, these changes do not appear to imply that there is a substantial difference in catalysis due to these differences (Table 3).

FIG 3 Structure of MERS-CoV PLpro bound to ChISG15. (A) Cartoon rendering of the MERS-CoV PLpro bound to ChISG15. The PLpro is shown in gold, and ChISG15 is shown in teal. The structural domains of MERS-CoV PLpro consist of the fingers (white), palm (lavender), thumb (green), and Ubl (pink) Domains. (B) Interactions at the interface between PLpro and ChISG15 colored as described for panel A. Hydrogen bonds are represented with black dashed lines, with distances labeled in black.

Intriguingly, the latter open state of MERS-CoV PLpro-Ub is more representative of the recently elucidated SARS-CoV PLpro-ChISG15 structure. This could indicate that, much like PLpro's interaction with Ub, more than one bound state of hISG15 in a PLpro may also exist (Fig. 5A). For the SARS-CoV PLpro, this may offer an explanation of how the remaining distance found in current models between the N-terminal domain of hISG15 and SARS-CoV PLpro's ridgeline helix residues closes (19). Specifically, the movement of the Zn fingers in the SARS-CoV PLpro to a similar position in MERS-CoV PLpro would require movement of the bound ChISG15 domain.

Naturally, the global differences between SARS-CoV PLpro and MERS-CoV PLpro-bound ChISG15 structures are reflected in specific amino acid interactions. Two of the hydrophobic interactions that drive the MERS-CoV PLpro's interaction with the ChISG15 are absent from the SARS-CoV PLpro and ChISG15 complex due to the more closed conformation of the zinc finger of the MERS-CoV PLpro. This closed conformation allows for one of the loops that coordinate the zinc to come in closer proximity to the ChISG15, creating a large hydrophobic seam. This conformation also allows for the interaction with the top of the thumb domain and the helix connecting the Ubl domain by the ChISG15, which creates the second hydrophobic interaction absent in the SARS-CoV PLpro-hISG15 interface (Fig. 5B, frame 1). In addition, the hydrophobic interaction between the Pro224 of the SARS-CoV PLpro and Phe149 of the ChISG15 is lost in the MERS-CoV complex due to the replacement with a valine, which is not close enough to make an interaction (Fig. 5B, frame 2). The only similarities between the two structures are that both proteases contain a hydrophobic interaction with Trp123, which is a highly conserved residue among ISG15s, and that Lys129 from the ChISG15 makes hydrogen bonding networks with residues, although different, in the protease (Fig. 5B, frames 3 and 4).

FIG 4 Comparison of ChISG15 and Ub binding by MERS-CoV. (A) Overlay of MERS-CoV PLpro-ChISG15 rendered as a cartoon with the open and closed conformations of MERS-CoV PLpro bound to Ub (pink and burgundy, respectively) (PDB accession number 4RF1, shown in yellow, and PDB 4RF0, shown in orange, respectively). The MERS-CoV in complex with ChISG15 is colored as described in the legend of Fig. 3A with the zinc finger shown in raspberry. The zinc atom is shown as a black sphere. (B) Close-up of ChISG15 and Ub recognition sequence (LRLRGG) in the MERS-CoV active site. The MERS-CoV in complex with ChISG15 is colored as described in the legend of Fig. 3A, and MERS-CoV in complex with Ub is shown in brown and burgundy, respectively.

Given that the PLpros from MERS-CoV and SARS-CoV engage ChISG15 through different interactions, this may provide insight into how the two proteases' preference for ISG15 from certain species can differ. For instance, MERS-CoV PLpro was shown to have minimal activity toward ISG15 from the northern tree shrew, whereas PLpro from SARS-CoV rapidly processed this ISG15 (19). With the additional structural information

TABLE 3 Kinetic analysis of MERS-CoV PLpro and MERS-CoV PLpro mutants K176E and V210D with ISG15-AMC and Ub-AMC^a

Protein and substrate	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)	k_{cat} (min^{-1})	K_m (μM)
MERS-CoV PLpro WT			
ISG15-AMC	7.13 ± 0.124	15.62 ± 0.510	2.19 ± 0.255
Ub-AMC	3.26 ± 0.0842	10.57 ± 0.270	3.24 ± 0.266
MERS-CoV PLpro K176E			
ISG15-AMC	0.74 ± 0.170^b		
Ub-AMC	3.85 ± 0.120	12.22 ± 0.430	3.17 ± 0.366
MERS-CoV PLpro V210D			
ISG15-AMC	0.23 ± 0.050^b		
Ub-AMC	2.93 ± 0.252	29.44 ± 2.40	10.04 ± 2.33

^aError was determined using standard deviation.
^bApproximate k_{cat}/K_m values were derived from the slope of the best-fit line of the data presented in Fig. 8B.

FIG 5 Comparison of ChISG15 and Ub binding by SARS-CoV. (A) Overlay of MERS-CoV PLpro-ChISG15 with SARS-CoV PLpro-ChISG15, shown as cartoons. MERS-CoV PLpro-ChISG15 is colored as described in the legend of Fig. 3A, with its zinc finger highlighted in light brown; SARS-CoV PLpro is colored according to the scheme for MERS, with its zinc finger highlighted in forest green, and ChISG15 from the SARS-CoV PLpro complex is shown in lavender. (B) Close-up interactions illustrating the differences in the way the PLpros accommodate ChISG15, with hydrogen bonds represented with black dashed lines. (C) Cleavage assay of MERS-CoV against wild-type shrew ISG15 and human-derived mutant shrew ISG15. N. shrew, northern tree shrew.

surrounding MERS-CoV PLpro, this lack of efficacy toward northern tree shrew ISG15 for MERS-CoV PLpro may originate from differences between northern tree shrew ISG15 and hISG15 at positions 144 and 145. Instead of proline and leucine residues at these positions, northern tree shrew ISG15 has a serine and glutamine, respectively (Fig. 5B, frame 2). Through swapping these residues out in northern shrew ISG15 and assessing their relevance using a proform ISG15 cleavage assay, a minor improvement of MERS-CoV PLpro for northern shrew ISG15 was observed. Specifically, a faint cleavage product was observed at the 30-min mark, in line with greater cleavage at the 60-min mark. However, this improvement was very minor, suggesting that additional differences between northern shrew and human ISG15s, to possibly include the N terminus, likely also play a factor in the species specificity of MERS-CoV PLpro.

N-terminal domain of hISG15. Despite the ITC affinity data between hISG15 and ChISG15 with MERS-CoV PLpro implying possibly minimal interactions of the N-terminal domain of hISG15 with MERS-CoV PLpro, the additional rotation of ChISG15 within the binding site of MERS-CoV PLpro suggested that the N-terminal domain of ISG15 might interact with MERS-CoV PLpro. To investigate this possibility, a model of MERS-CoV PLpro bound to hISG15 using an hISG15 structure (PDB accession number 1Z2M) with ChISG15 as an anchor was generated. Within this model, a number of residues potentially pointed toward where the N-terminal domain of hISG15 could be located (Fig. 6A). To probe potential interaction of these residues with hISG15, the activity of

FIG 6 Model of full-length ISG15 binding to MERS-CoV PLpro. (A) The C-terminal domain from full-length hISG15 (PDB accession number 1Z2M; light green), rendered as a cartoon, was aligned by the least squares (LSQ) method using the ChISG15 from the MERS-CoV PLpro-ChISG15 structure as an anchor. The PLpro is shown as a cartoon with a transparent surface rendering, colored as described in the legend of Fig. 3A. Mutation sites in the ridgeline helix are labeled in purple and shown as lavender sticks. (B) Enzyme activity of the ridgeline mutants, relative to that of the wild type. PEP, peptide.

wild-type MERS-CoV PLpro as well as MERS-CoV PLpro containing disruptive mutations at those sites was assessed using three substrates. These were Ub-AMC, ISG15-AMC, and the small peptide Z-RLRG-AMC, which are designed utilizing the 7-amino-4-methylcoumarin (AMC) leaving group. The small peptide represents the consensus sequence for both Ub and ISG15 and is used as a control to ensure that intrinsic catalytic activity is not significantly impacted. Although this set even contained some sites homologous to SARS-CoV PLpro's ridge helix, which were previously implicated in interacting with the N-terminal domain of hISG15, the potentially disruptive mutations failed to reduce deISGylase activity (Fig. 6B). Intriguingly, one mutation, E70A, located in the Ub-like domain, had a significant increase in deISGylase activity, implying that residues in the Ubl potentially could play a role in some CoV PLpro-ISG15 interactions.

Structurally guided mutations to reduce deISGylase activity. One of the primary obstacles to observing the impact and role that viral deISGylase activity plays in viral host evasion and pathogenesis has been the inability to create attenuated viruses lacking this function, specifically, creating an attenuated PLP that decreases viral deISGylase activity while not significantly altering its other functionalities. Utilizing the newly available structural information related to the MERS-CoV PLpro-ChISG15 binding interface, along with ITC and mutational data advocating the C-terminal domain as the principle binding domain, MERS-CoV PLpros with attenuated deISGylase activity were sought. Specifically, 11 mutations were designed for their potential to selectively disrupt the interface between MERS-CoV PLpro and hISG15 without appreciably impacting deubiquitinase and peptidase activities (Fig. 7A and B).

Four of these mutations, V225D, V225A, Q227A, and R232V, had no substantial impact on any activity. However, one mutation, V225P, unexpectedly increased activity

FIG 7 Structurally guided mutations utilizing the MERS-CoV PLpro bound to hISG15. (A) Mutated residues are highlighted based on activity; mutants shown in green have reduced activity toward ISG15-AMC, mutants shown in cyan have increased activity toward ISG15-AMC, the mutant shown in magenta showed little change in activity, and the mutant shown in gray increased activity toward Ub-AMC. hISG15 and Ub from the closed conformation of MERS-CoV PLpro-Ub complex, rendered as ribbons, were overlaid based on an alignment with the MERS-CoV PLpros and colored as described in the legends of Fig. 3A and 4A. In the inset, MERS PLpro's interaction surface with ISG15 and Ub is highlighted in teal and burgundy, respectively. (B) Enzyme activity of the mutants, at the interface, relative to that of the wild type. (C) Close-up interactions of mutants that have an overall effect on ISG15-AMC and/or Ub-AMC activity, with the electrostatic interactions represented with black dashed lines. In frame 2, R232 from the closed conformation of MERS-CoV PLpro bound to Ub is shown in lilac. (D) Close-up interactions of mutants that specifically reduce activity toward ISG15-AMC, with electrostatic interactions represented with black dashed lines.

toward both Ub-AMC and ISG15-AMC (Fig. 7B). For V225P, the original desired effect was to alter the size of the hydrophobic residue in a manner to discourage interaction with ISG15's Leu85 while encouraging tighter interactions with Ub's Phe4 (Fig. 7C, frame 1). The increase of over 80% in activity toward Ub-AMC and ISG15-AMC implies that this strategy was ineffective (Fig. 7B). One explanation might be that this mutation increased hydrophobic interactions of the protease with both substrates. A mutation having the opposite effect was R232A. The R232A mutation was designed to take advantage of a H-bond interaction between the protease and hISG15 that is not

present with Ub (Fig. 7C, frame 2). This mutation resulted in an ~50% decrease in both delSGylase and deubiquitinase activities (Fig. 7B). As a result, this appears to imply that the salt bridge formed between Arg232 and Glu189 observed in the Zn finger of the MERS-CoV PLpro-Ub structure is key in the protease's engagement of Ub. Another mutation that gave a surprising result is R234A. Although it did give a slight decrease in activity toward ISG15-AMC, a significant increase in activity toward Ub-AMC was observed (Fig. 7B). This mutation was initially designed to disrupt salt bridge interactions between MERS-CoV PLpro's Arg234 and hISG15's Glu127 (Fig. 7C, frame 3). In light of the increased protease activity toward Ub-AMC, this alteration inadvertently boosted protease activity toward Ub-AMC. A possible explanation for this unexpected activity could be that the removal of the charged residue leaves a predominantly hydrophobic surface. This surface may be more likely to interact with the opposing hydrophobic surface of Ub (Fig. 7C, frame 3).

Intriguingly, the most successful mutations to remove delSGylase activity occurred at positions 176 and 210. Mutations K176L, K176E, and K176A showed a significant decrease in ISG15 with little to no change in overall Ub activity (Fig. 7B). These mutations were designed to disrupt the salt bridge formed by Lys176 with hISG15's Glu132 (Fig. 7D, frame 1). This interaction is not observed between Ub and the protease due to the differences in the orientations of both residues. As a result, loss of the electrostatic interaction, for K176L and K176A, or introduction of an electrostatic repulsion, for K176E, reduced the ability of the protease to engage hISG15. Of these, the electrostatic repulsion appeared to be the most effective at squelching delSGylase activity with little impact on other functionalities (Fig. 7B). In addition to Lys176, the mutation of Val210 to an aspartic acid was the most successful in eliminating delSGylase activity. The introduction of aspartic acid was designed to take advantage of the differences at Ub's Arg42, Ile44, and Gln49 and hISG15's Trp123, Thr125, and Pro130 residues, respectively (Fig. 7D, frame 2). The V210D mutation creates a steric hindrance and electrostatic repulsion with Trp123 of hISG15. There was a concern that this might also disrupt the nearby hydrophobic path involving Ub's Ile44. However, the deubiquitinase activity remains near wild-type levels, suggesting either that this does not occur or that perhaps any disruption is offset with the formation of other interactions with nearby residues, possibly Arg42. The overall effect of the V210D mutation is a reduction of delSGylase activity to 4% (Fig. 7B).

With V210D and K176E offering the most promising options to eliminate delSGylase activity without disrupting catalysis and retaining deubiquitinase activity, their impact on MERS-CoV function was further examined. Utilizing an assay for the pro-hISG15, the effects of V210D and K176E are considerable. Whereas MERS-CoV PLpro typically cleaves pro-hISG15 almost completely after 10 min, no complete cleavage of pro-hISG15 is observed for K176E after 60 min (Fig. 8A). To gain insights on the effects of these mutations on binding Ub and ISG15, ITC was performed. Interestingly, no binding event was observed between either mutant and hISG15 (data not shown). In contrast, binding was detected with K176E and V210D for Ub. In detail, the K176E exhibited a dissociation constant of $7.41 \pm 0.643 \mu\text{M}$ that mirrored that of wild-type MERS-CoV PLpro. The V210D mutant exhibited weaker binding to Ub, with a K_D of $200.50 \pm 7.77 \mu\text{M}$, that could be the source of the ~20% reduction of deubiquitinase activity observed for V210D during the $1 \mu\text{M}$ Ub-AMC assay (Table 1 and Fig. 2B). To provide further insight into the effects of the mutants, enzyme kinetic analysis of K176E and V210D mutants was performed. The mutant K176E possessed a K_m of $3.17 \pm 0.366 \mu\text{M}$ and k_{cat} of $12.22 \pm 0.430 \mu\text{M}^{-1} \text{min}^{-1}$ for Ub that are virtually indistinguishable from the values of the wild-type MERS-CoV. The mutant V210D had a 3-fold elevated K_m of 10.04 ± 2.33 , echoing the weaker binding of Ub observed on the ITC. However, it also possessed a 3-fold higher k_{cat} of $29.44 \pm 2.40 \mu\text{M}^{-1} \text{min}^{-1}$ (Fig. 8B). As a result, the overall catalytic efficiencies for V210D and K176E were nearly identical to the efficiency of wild-type MERS-CoV for the Ub substrate (Table 3). In stark contrast to the Ub substrate, both V210D and K176E were unable to be saturated with the hISG15

FIG 8 Analysis of MERS-CoV PLpro mutants K176E and V210D. (A) Cleavage assay of MERS-CoV PLpro and MERS-CoV PLpro mutants K176E and V210D against pro-hISG15. (B) Graphs of steady-state kinetics for MERS-CoV PLpro (red square) and MERS-CoV PLpro mutants K176E (blue triangle) and V210D (green circle) for ISG15- and Ub-AMC. Error was calculated using the standard deviation.

substrate. Therefore, their apparent k_{cat}/K_m values were calculated from the slope of the line in Fig. 8B. The mutants K176E and V210D had a 5-fold and one order of magnitude decrease of catalytic efficiency compared to wild-type MERS-CoV levels, respectively (Table 3).

In perspective, V210D's k_{cat}/K_m of $0.23 \pm 0.050 \mu\text{M}^{-1} \text{min}^{-1}$ and K176E's k_{cat}/K_m of $0.74 \pm 0.170 \mu\text{M}^{-1} \text{min}^{-1}$ for the hISG15 substrate were more reflective of the k_{cat}/K_m of 0.3 ± 0.1 reported for the SARS-CoV PLpro catalytic efficacy of the peptide-AMC (21). Additionally, a similar k_{cat}/K_m of $0.33 \pm 0.066 \mu\text{M}^{-1} \text{min}^{-1}$ was observed for a Crimean-Congo hemorrhagic fever viral ovarian tumor domain (CCHFV vOTU) P77D mutant that had a significantly impaired ability to cleave cellular ISGylated substrates (22). As a result, the identification of the V210D and K176L/A/E mutants offers new tools for use in reverse genetic systems to probe how the removal of viral deISGylase activity impacts the pathogenesis of MERS-CoV and other deISGylase-containing viruses.

MATERIALS AND METHODS

Expression and purification of MERS-CoV PLpro. MERS-CoV PLpro in pET-15b was expressed as previously described (19). Purification was adapted from the previously described method (19). Briefly, cells expressing the recombinant protein were lysed in 500 mM NaCl, 50 mM Tris (pH 7.0), and 10 mM β -mercaptoethanol (BME) for 30 min at 4°C. The cells were subsequently sonicated on ice at 50% power, with a 50% duty cycle for a total of 6 min. The lysate was subjected to centrifugation at $70,600 \times g$ for 30 min, and the soluble fraction was collected and filtered through a 0.8- μm -pore-size filter. The protein was then poured over high-density nickel agarose beads (Gold Biotechnology, Olivette, MO) preequilibrated with 500 mM NaCl and 50 mM Tris (pH 7.0), followed by a column wash with 10 column volumes of buffer supplemented with 10 mM imidazole and elution with 10 column volumes of buffer supplemented with 300 mM imidazole. The eluted protein was further purified using a Superdex 200 column (GE Healthcare, Pittsburgh, PA) preequilibrated with 100 mM NaCl, 5 mM HEPES (pH 7.0), 2 mM dithiothreitol (DTT), and 0.1 mM ZnCl₂.

ITC of MERS-CoV PLpro binding with ChISG15, hISG15, and Ub. Expression and purification of ChISG15, hISG15, and Ub in pET-15b for ITC were done using a previously described protocol (23). ITC measurements of MERS-CoV PLpros with ChISG15 or Ub were performed according to a previously published method (19). To summarize, ITC was performed using a Microcal PEAQ-ITC (Malvern, Worcestershire, United Kingdom). A series of 19 injections spaced at 180 s apart at 2 μl per injection was used. All injections were kept at a constant temperature of 25°C utilizing a reference power of 4 $\mu\text{cal/s}$. Both the protease and substrate were dialyzed into 50 mM HEPES (pH 7.3), 200 mM NaCl, 1 mM DTT, and 0.1 mM ZnCl₂ overnight at 4°C. For MERS-CoV PLpros and ChISG15, the protease was placed in the unit cell at 277 μM with ChISG15 in the syringe at 2.76 mM. This experiment was run five times. For MERS-CoV PLpros and either hISG15 or Ub, the protease was placed in the unit cell at 255 μM with the ligand in the syringe at 2.58 mM. These experiments were run in duplicate. The data were processed using Microcal PEAQ-ITC analysis software.

MERS-CoV PLpro-ChISG15 complex formation and purification. ChISG15 in the vector pTYB2 was expressed, purified, and derivatized as previously described (23). Complexation of the PLpro with the propargylamine-derivatized ChISG15 (ChISG15-PA) was adapted from a previously published method

(19). In brief, approximately equimolar quantities of MERS-CoV PLpro and ChISG15-PA were mixed and incubated overnight at 4°C. The mixture was then dialyzed into 250 mM NaCl–25 mM HEPES (pH 7.0) for 4 h, filtered through a 0.8- μ m-pore-size filter, and poured over a nickel agarose column to eliminate uncomplexed ChISG15. The column was washed with 10 column volumes (CV) of buffer supplemented with 10 mM imidazole, and the complex was eluted with 10 CV of buffer supplemented with 300 mM imidazole. The eluted complex was dialyzed in 250 mM NaCl, 25 mM HEPES (pH 7.0), 2 mM DTT, and 0.1 mM ZnCl₂ overnight with thrombin to cleave a 6 $\times$ His tag. The thrombin-cleaved complex was further purified by size exclusion chromatography using a Superdex 75 column (GE Healthcare, Pittsburgh, PA) pre-equilibrated with 100 mM NaCl, 5 mM HEPES (pH 7.0), 2 mM DTT, and 0.1 mM ZnCl₂.

Crystallization of MERS-CoV PLpro-ChISG15 complex. The purified complex was concentrated to 12.2 mg/ml and screened against a range of conditions in a Qiagen NeXtal suite by the hanging drop method using a TTP Labtech Mosquito (TTP Labtech, Hertfordshire, United Kingdom). The initial screen yielded the best crystals in a condition containing 200 mM CaCl₂, 100 mM NaO₂C₂H₃ (pH 4.6), and 30% (vol/vol) 2-methyl-2,4-pentanediol (MPD). This condition was optimized using salt and precipitant gradients. The final optimized condition consisted of 200 mM CaCl₂, 100 mM NaO₂C₂H₃ (pH 4.6), and 28% (vol/vol) MPD. The final crystals for data collection were obtained by vapor diffusion using a 500- μ l reservoir solution with 2- μ l hanging drops consisting of protein and reservoir solution mixed in a 1:1 ratio.

Data collection, processing, and structure refinement. Crystals of MERS-CoV PLpro-ChISG15 were soaked in mother liquor as a cryoprotectant and flash-frozen in liquid N₂. Native and zinc single anomalous dispersion (Zn-SAD) data sets were collected at wavelengths of 1.0 Å and 1.2837 Å, respectively, using the 22-ID beamline at Argonne National Labs. Data sets were collected at 100 K. Collected data sets were indexed, integrated, and scaled using HKL-2000 (24). Experimental phasing was employed using the Phenix suite of programs. In detail, a hybrid substructure search was used to find Zn sites, initially identifying seven, with Phaser solving the experimental phases (25, 26). Thereafter, auto-build and refinement using Coot and Phenix generated a search model that was used for molecular replacement into the 2.4-Å native data set using Phaser. Subsequently, alternating rounds of model building and refinement in Coot and Phenix were used to generate the final structure (27–30). The final structure was modeled with six Zn ions, with two occupying the Zn ribbon motifs of the proteases and four involved in crystal packing between the proteases. The refined structure was validated using Molprobit (31). Statistics for data collection and refinement are presented in Table 2.

Generation of MERS-CoV PLpro and shrew pro-form ISG15 (proISG15) mutants. Mutations were introduced using a QuikChange Lightning kit according to the manufacturer's protocol (Agilent Technologies, Inc.). The resultant plasmids were transformed into *Escherichia coli* NEB-5 α cells by heat shock. Mutant plasmids were confirmed by sequencing and transformed into BL21(DE3) cells.

Mutational analysis of MERS-CoV PLpro enzymatic activity. Assessment of purified wild-type and mutant MERS-CoV PLpro activity was adapted from a previously described method (32). Briefly, each protease was tested against commercially available fluorogenic substrates of Ub, ISG15, and a peptide representing the shared C-terminal consensus sequence, Z-RLRGG, conjugated to 7-amino-4-methylcoumarin (AMC). All assays were performed in duplicate at 25°C in 100 mM NaCl, 50 mM HEPES (pH 7.5), 0.01 mg/ml bovine serum albumin, and 5 mM DTT in a 50- μ l reaction volume using 96-well Corning Costar half-volume plates. For assays with Ub- and ISG15-AMC (Boston Biochem, MA), the enzyme was present at 5 nM, and the substrate was present at 1 μ M. For Z-RLRGG-AMC (Bachem), the assay was run with 1 μ M enzyme and 50 μ M substrate. Fluorescence produced by cleavage of AMC from the substrate was measured using a CLARIOstar plate reader (BMG Labtech, Inc.), and the data were analyzed using MARS (BMG Labtech, Inc.). Enzyme turnover rates were calculated utilizing steady-state kinetics.

Kinetic characterization of Ub-AMC and ISG15-AMC cleavage by MERS-CoV PLpro WT and V210D and K176E mutants. Further kinetic analysis for MERS-CoV PLpro and the V210D and K176E mutants was performed as for the initial assays with some modifications. Assays were run with 5 nM protease in a 30- μ l reaction volume with concentrations of Ub- and ISG15-AMC varying from 0.25 μ M to 20 μ M. For MERS-CoV PLpro V210D with Ub-AMC, an additional concentration was run at 50 μ M. Reactions were initiated by the addition of protease. The initial reaction rates at each concentration of substrate were plotted and analyzed based on Michaelis-Menten kinetics using the enzyme kinetics software of SigmaPlot (version 12; Systat Software, Inc.). The plotted curves are shown in Fig. 8, with values derived from the respective curves shown in Table 3. In the case of K176E with ISG15-AMC and V210D with ISG15-AMC and Ub-AMC, for which the reaction rates did not approach saturation, apparent k_{cat}/K_m values were calculated based on linear regression of the initial velocity as a function of substrate concentration (21).

Cleavage of proISG15 by MERS-CoV PLpro. Shrew proISG15 mutants were expressed and purified based on a previously reported protocol (23). Cleavage assays of human proISG15 with MERS-CoV PLpro V210D and K176E mutants, respectively, and shrew mutants S144P and Q145L with MERS-CoV PLpro WT were performed as previously described (19, 23). Briefly, reactions were run in 100 mM NaCl, 5 mM HEPES (pH 7.5), and 2 mM DTT at 37°C with 20 nM protease and 10 μ M proISG15. Samples were taken at time points up to 1 h, and the reaction was quenched by mixing equal parts with 2 $\times$ SDS-tricine sample buffer and boiling at 98°C for 5 min. Samples were subsequently analyzed by SDS-PAGE on 10 to 20% Mini-Protean Tris-tricine precast gels (Bio-Rad, Hercules, CA).

Accession number(s). Protein structures of the MERS-CoV PLpro were deposited in the Protein Data Bank under PDB IDs [5W8T](#) and [5W8U](#).

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