Macro Domain from Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is an Efficient ADP-ribose Binding Module: Crystal Structure and Biochemical Studies

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ABSTRACT
The newly emerging Middle East respiratory syndrome coronavirus (MERS-CoV) encodes the conserved macro domain within non-structural protein 3. However, the precise biochemical function and structure of the macro domain is unclear. Using differential scanning fluorimetry and isothermal titration calorimetry, we characterized the MERS-CoV macro domain as a more efficient adenosine diphosphate (ADP)-ribose binding module than macro domains from other CoVs. Furthermore, the crystal structure of MERS-CoV macro domain was determined at 1.43-Å resolution in complex with ADP-ribose. Comparison of macro domains from MERS-CoV and other human CoVs revealed structural differences in the α1 helix alters how the conserved Asp20 interacts with ADP-ribose and may explain the efficient binding of the MERS-CoV macro domain to ADP-ribose. This study provides structural and biophysical bases to further evaluate the role of the MERS-CoV macro domain in the host response via ADP-ribose binding but also as a potential target for drug design.

INTRODUCTION
Since the severe acute respiratory syndrome (SARS) outbreak, in 2003 (1,2), a newly discovered disease, Middle East respiratory syndrome (MERS), has been spreading from countries in the Middle East to America (3-5). In the summer of 2015, MERS was reported in North East Asia (6-8). The causative agent of MERS was identified as an unknown coronavirus (CoV) resembling SARS-CoV and referred to as Middle East respiratory syndrome CoV (MERS-CoV) (9-12). MERS-CoV belongs to the genus Betacoronavirus and possesses a positive-strand RNA genome that encodes viral proteins essential to the life cycle of the virus (13,14). The mortality of MERS is fourfold higher than SARS (40% compared to 10%) (15). Since the first case report in Saudi Arabia, MERS has been reported in more than 20 countries and has caused more than 400 deaths worldwide (9).

CoVs utilize the RNA genome to encode structural proteins, including spike glycoprotein (S), membrane protein (M), and nucleocapsid protein (N). They encode a large number of non-structural proteins (NSPs) for rapid replication. A single large replicase gene
encodes all proteins involved in viral replication. The replicase gene contains two open reading frames (ORFs), ORF1a and ORF1b, which encode two polyproteins, pp1a and pp1ab; production of pp1ab requires a ribosomal frameshift to transcribe the portion encoded by ORF1b (16). ORF1a encodes viral proteases – main protease (Mpro, also called 3CLpro) and papain-like protease (PLpro) – that are responsible for cleavage of the ORF1a and ORF1b gene products to produce functional NSPs.

In SARS-CoV, the largest NSP member, NSP3, is a multidomain protein containing the following domains: N-terminal acidic domain, macro domain, SARS-unique domain, PLpro, nucleic acid-binding domain, marker domain (G2M), transmembrane domain (TM), and Y-domain (17). The MERS-CoV genome contains 16 NSPs (Figure 1); except for 3CLpro and PLpro (18,19), most of the functional domains within the NSP3 in MERS-CoV remain structurally uncharacterized.

The macro domain is named after the non-histone motif of the histone variant macroH2A, in which it was originally characterized (20-22), a protein module ubiquitous in eukaryotes, bacteria, and archaea. This domain is well known for its affinity to adenosine diphosphate (ADP)-ribose (23-25). Many cellular enzymes bearing macro domains within their structures interact with poly ADP-ribose (PAR) (26-29). Poly ADP-ribosylation (PARylation) is a post-translational modification linked with DNA repair, apoptosis, gene regulation, and protein degradation. Thus, macro domain-containing proteins and enzymes may play important roles in regulating various cellular processes (30).

Surprisingly, the CoVs studied so far and a few other viruses such as alphavirus, rubella virus, and hepatitis E virus possess macro domains in their genomes (16). In addition, some viral macro domains were found to have ADP-ribose 1’-phosphate phosphatase (ADRP) activity (31-33), which catalyzes the removal of phosphate from ADP-ribose 1’-phosphate (Appr1p) to produce ADP-ribose. ADRP activity has been reported in a yeast protein containing macro domain as well as AF1521 protein in Archaeoglobus fulgidus (23,34,35). The enzymatic activity of viral macro domains in processing Appr1p is low (33,36-38) and appears to be dispensable for virus RNA synthesis (31). In addition, the mutant for the CoV mouse hepatitis virus A59 (MHV-A59), encoding a single amino-acid substitution of a strictly conserved residue for ADRP activity, replicated to slightly reduced titers in mouse liver but, strikingly, did not induce liver disease (39). The MHV macro domain exacerbates MHV-induced liver pathology, most likely by inducing excessive inflammatory cytokine expression. It was also reported that catalytic residues Asn809, His812, Gly816 and Gly817 for ADRP activity in hepatitis E virus (HEV) macro domain are critical for HEV replication (40). Accordingly, the development of drugs targeting the viral macro domain may be a strategy for anti-viral therapy.

The macro domain of SARS-CoV NSP3 was previously reported to possess ADP-ribose and PAR binding ability, which suggests that the macro domain may regulate cellular proteins involved in an apoptotic pathway via PARylation to mediate the host response to
infection (36). Structural studies of macro domains from CoVs such as human CoV 229E (HCoV-229E) and feline CoV (FCoV) also revealed interactions with ADP-ribose (41-43) and have offered huge advances in our understanding of viral macro domains. The MERS-CoV genome features a macro domain embedded in NSP3 (Figure 1). However, we lack structural and functional information regarding the MERS-CoV macro domain.

In the present study, we investigated the MERS-CoV macro domain as an ADP-ribose binding module, with comparison to previously characterized viral macro domains. Furthermore, we determined the crystal structure of the MERS-CoV macro domain in complex with ADP-ribose. Structural comparison of MERS-CoV and other human CoVs revealed divergence in ADP-ribose binding by macro domains. Our study may shed new light on structurally based design of novel antiviral drugs targeting viral macro domains.

EXPERIMENTAL PROCEDURES

Protein expression and purification

The DNA sequence containing the MERS-CoV macro domain was synthesized by a local biotechnology company (MDBio, Inc.) and cloned into the pUC57 plasmid. The macro domain fragment was inserted between the NdeI and XhoI sites of the pET28a vector system (Novagen). The forward and reverse PCR primers used for amplification were macro-F (5'-AATTCCATATGCCACTGAGCAATTTTGAACA-3') and macro-R (5'-AATTCTCGAGTATGATGTCAGGCTCTCT). The resulting plasmid with the inserted sequence was transformed into *Escherichia coli* BL21(DE3) cells, which were grown at 37°C up to OD$_{600}$ 1.0 with 50 μg/mL kanamycin. The expression of the recombinant MERS-CoV macro domain with an His-tag at the N terminus was induced in cells with 1 mM isopropyl-β-D-thiogalactoside, followed by growth for 20 h at 16°C. Cells were collected by centrifugation and resuspended in lysis buffer (25 mM phosphate buffer, pH 7.0, 100 mM NaCl). After 20 min of sonication, the cell extract was clarified by centrifugation at 18,900 × g for 30 min at 4°C to remove debris. The clear supernatant was placed in an open column filled with Ni$^{2+}$-NTA resin. The resin was washed with ten times volume of lysis buffer containing 50 and 100 mM imidazole, respectively. The His-tagged MERS-CoV macro domain was eluted by lysis buffer containing 300 mM imidazole. The purified MERS-CoV macro domain was dialyzed against stabilization buffer (25 mM phosphate buffer, pH 7.0, 100 mM NaCl, 0.5 mM dithiothreitol). The His-tag was removed by using thrombin, which resulted in four additional residues (GSHM) at the N terminus. The protein was further purified by gel filtration chromatography with a Superdex75 XK 16/60 column (GE Healthcare) in 20 mM Tris-HCl buffer (pH 7.0), 100 mM NaCl.

Circular dichroism (CD) spectroscopy

Far-UV CD spectra were measured with 10-μM protein samples in CD buffer (20 mM phosphate buffer, pH 3.5-8.5) placed into a 1-mm pathlength cuvette and recorded on a JASCO J-810 spectropolarimeter equipped with a Peltier
temperature control system (JASCO International Co.). Thermal transition of protein samples with or without pre-incubation of 1 mM ADP-ribose were monitored at 220 nm from 25°C to 95°C at a scan rate of 1°C/min. Baseline subtraction, smoothing and data normalization involved use of SigmaPlot. The melting temperature (Tm) was calculated with the maximum of the first derivative of the CD signal.

Differential scanning fluorimetry (DSF)

Thermal shift assay with DSF involved use of a CFX48 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). In total, 25 μL mixture containing 2 μL SYPRO Orange (Sigma-Aldrich), 1.25 μL dialysis buffer (20 mM Tris-HCl, and 100 mM NaCl, pH 7.0), 10 μL of 1 μM protein sample, and various concentrations of ADP-ribose were mixed on ice in an 8-well PCR tube. Fluorescent signals were measured from 25°C to 95°C in 0.1°C/30-sec steps (excitation, 450-490 nm; detection, 560-580 nm). The main measurements were carried out in triplicate. Data evaluation and Tm determination involved use of Bio-Rad CFX Manager, and data fitting and dissociation constant (Kd) calculation involved use of SigmaPlot.

Isothermal titration calorimetry (ITC)

Binding of ADP-ribose to the MERS-CoV macro domain was measured by ITC with the Nano Isothermal Titration Calorimeter (TA Instruments). Aliquots of 3 μL of 1.14 mM ADP-ribose were titrated by injection into protein (0.057 mM in 0.98 mL) in 20 mM Tris-HCl (pH 7.0) and 100 mM NaCl. Experiments were carried out at 25°C with 250 rpm stirring. Background heat from ligand to buffer titrations was subtracted, and the corrected heat from the binding reaction was used to derive values for the stoichiometry of the binding (n), association constant (Ka), Kd, apparent enthalpy of binding (ΔH), and entropy change (ΔS). Data were fitted by use of an independent binding model with Launch NanoAnalyze v2.3.6.

Crystallization and data collection

The MERS-CoV macro domain and ADP-ribose were mixed in a molar ratio of 1:15. Initial protein crystallization trials were performed at 283 K by the sitting-drop vapour-diffusion method with commercial crystallization screen kits, 96-well Intelli-plates (Art Robbins Instruments) and a HoneyBee 963 robot (Genomic Solutions). Each crystallization drop was prepared by mixing 0.3 μL macro domain/ADP-ribose at 10 mg/mL with an equal volume of mother liquor, and the mixture was equilibrated against 100 μL reservoir solution. The crystals for data collection were grown in 1 week at 283 K with the optimal condition of 100 mM phosphate-citrate pH 4.2, 2.0 M ammonium sulfate, and 10 mM nicotinamide adenine dinucleotide as the additive. For subsequent anomalous phasing, the crystal was soaked for 8 hr in 3 mM mercuric chloride, cryoprotected in mother liquor supplemented with 20% glycerol, and flash-frozen in liquid nitrogen at 100 K. The diffraction images were recorded in a 100-K nitrogen gas stream with use of BL13B1 or


BL13C1 beamlines (National Synchrotron Radiation Research Center, Taiwan) and processed by using HKL2000 software (44).

Structure determination and refinement

The crystal structure of MERS-CoV macro domain in complex with ADP-ribose was solved by the Hg-derivative single-wavelength anomalous dispersion (SAD) method by using SHELXD/SHELXE software (45). The initial model was refined by the maximum likelihood method implemented in REFMAC5 (46) as part of the CCP4 suite (47) and rebuilt interactively by inspecting the σ-weighted electron density maps with coefficients $2mF_o-DF_c$ and $mF_o-DF_c$ in COOT (48). During the later stages, restrained positional and B-factor refinement involved the program phenix.refine (49). Water molecules were manually added at the final stages. The models were evaluated with use of PROCHECK (50) and MOLPROBITY (51). The data collection and structure refinement statistics are in Table 1.

RESULTS AND DISCUSSION

ADP-ribose binding ability of MERS-CoV macro domain

The MERS-CoV macro domain (pp1a residues 1110 to 1273) was expressed and purified from E. coli. The final purified protein was a 167-amin acid protein (20 kDa), with four additional residues at the N-terminus resulting from removal of the hexa-histidine tag after thrombin cleavage. CD spectra revealed that the macro domain exhibited a stable α/β type folding pattern under various pH conditions (Figure 2). The Tm of the macro domain from thermal transition monitored by CD was 43°C. However, the addition of ADP-ribose significantly increased the Tm to 51°C (Figure 3A). The significant increase in Tm suggests the interaction between the MERS-CoV macro domain and ADP-ribose.

To understand the kinetics of ADP-ribose binding to the MERS-CoV macro domain, both DSF and ITC measurements were used to examine the equilibrium dissociation constant (Kd) of ADP-ribose. After fitting DSF data, the Kd was determined to be $3.12 \pm 0.42 \mu M$ ($r^2=0.9628$) (Figure 3B), which is similar to the calculated Kd of 2.95 μM based on ITC data (Figure 3C). In addition, ITC data indicated that ADP-ribose bound to the MERS-CoV macro domain with favorable enthalpy change (exothermic, $\Delta H=-91.04$ KJ/mol). The binding reaction was spontaneous at 25 °C with exergonic Gibbs energy of binding ($\Delta G=-31.56$ KJ/mol). The thermodynamic profile ($\Delta G<0$, $\Delta H<0$, and $-T\Delta S>0$) of ADP-ribose binding to the MERS-CoV macro domain suggests that ADP-ribose is likely stabilized by hydrogen bond formations (52).

We reviewed the results of previously reported binding assays of ADP-ribose binding to CoV macro domains (Table 2). Compared with the Kd of ADP-ribose binding to macro domains of human CoVs such as SARS-CoV (24 μM) (36) and HCoV-229E (28.9 μM) (41) and animal coronaviruses such as FCoV (~400 μM) (42), our Kd of 2.95 μM from biochemical analysis suggests that the MERS-CoV macro domain is a more efficient ADP-ribose binding module. The SARS macro domain possesses poly
ADP-ribose binding ability and may play a role in the host response to virus (36). We found that the MERS-CoV macro domain interacts with ADP-ribose, which suggests further investigating the role of the macro domain in MERS-CoV infection.

**Overall structure of MERS-CoV macro domain in complex with ADP-ribose**

We determined the crystal structure of ADP-ribose–bound MERS-CoV macro domain for further molecular elucidation. The orthorhombic crystals gave good-quality X-ray diffraction and belonged to the space group C222₁ with the following unit cell dimensions: a=41.798 Å, b=120.807 Å, c=67.659 Å, and α=β=γ=90°. The structure of the MERS-CoV macro domain was solved by Hg-SAD (see Experimental Procedures). The final protein structure (Figure 4A) was refined to 1.43 Å resolution with R-factor and R-free values of 0.1273 and 0.1619, respectively (Table 1). The core of the structure of MERS-CoV macro domain is a seven-stranded β-sheet in the order of β₁-β₂-β₇-β₆-β₃-β₅-β₄ (Figure 4B). The central β-sheet is sandwiched between six α-helices, with α₁, α₂ and α₃ packing onto one face and α₄, α₅ and α₆ onto the other. In the initial refinement cycle, a strong bent electron density (continuous at 1σ cutoff) (Figure 4A) located at the central pocket, was unambiguously identified as an ADP-ribose molecule. This molecule is tightly bound in an uncharged crevice located at the C-terminal end of strands β₃ and β₆ in the loop regions between β₃-α₂ and β₆-α₅ (Figure 4A).

A search of the DALI database (53) with the structure of the MERS-CoV macro domain in complex with ADP-ribose used as a model revealed several structural homologs. Top-ranked structures were macro domains of CoVs in complex with ADP-ribose such as those for SARS-CoV (PDB code, 2FAV; Z score 27.9; root-mean-square deviation [RMSD] 1.3; sequence identity 45%; sequence similarity 65%) (36), HCoV-229E (PDB code, 3EWR; Z score 22.8; RMSD 1.8; sequence identity 33%; sequence similarity 56%) (43), FCoV (PDB code, 3JZT; Z score 22.6; RMSD 1.8; sequence identity 30%; sequence similarity 53%) (42), and infectious bronchitis virus (PDB code, 3EWP; Z score 19.5; RMSD 1.9; sequence identity 28%; sequence similarity 47%) (43). This finding reflects that the viral macro domains are structurally well conserved. However, variability between all these structures arises from the loops connecting the core secondary structure elements, which display great diversity in sequence, length, and conformation and may correspond to different ADP-ribose binding ability.

**Molecular basis of ADP-ribose binding in MERS-CoV macro domain**

To gain insights into the molecular mechanism of ADP-ribose binding, we further investigated the binding pocket for ADP-ribose in the MERS-CoV macro domain. The adenine moity resides in the hydrophobic cavity containing Gly19, Ala21, Ile47, Pro123, Leu124 and Val152 (Figure 5A). Coordination of ADP-ribose involves serial hydrogen bond formations and hydrophobic interactions provided by surrounding amino acid residues.
(Figure 5B). The side chain of Asp20 contacts the N6 atom of the pyrimidine ring in adenine moiety via direct hydrogen bonding. This residue is critical for binding specificity of the macro domain AF1521 in *Archaeoglobus fulgidus* (54). Structure-based multiple sequence alignment showed that this aspartic acid is conserved among CoV macro domains (Figure 6A). Oxygen atoms of the pyrophosphate in ADP-ribose contact surrounding residues via hydrogen bonding with nitrogen atoms in backbone amides of Ile46, Ser126, Gly128, Ile129 and Phe130. The second ribose is stabilized by complex hydrogen bonding with surrounding residues and water molecules. The ribose-3" oxygen atom forms a hydrogen bond with a nitrogen atom in the side chain of Asn38. The ribose-2" oxygen atom forms hydrogen bonds with the oxygen atom in the side chain of Lys42 and the nitrogen atom in the backbone amide of Gly43. The ribose-1" oxygen atom forms a hydrogen bond with the nitrogen atom in the side chain of Gly45. A water molecule serves as a bridge between the ribose-1" oxygen atom, Asn38 and His43. This organization of the terminal ribose and surrounding molecules was also observed in the yeast ADRP enzyme (34), which suggests that Asn38 and His43 may be critical for the hydrolysis reaction of ADP-ribose 1"-phosphate to ADP-ribose. In addition, equivalent residues critical for ADRP activity in the SARS-CoV macro domain (36) included Asn35, Asn38, His43, Gly44, Gly45 and Phe130, which are conserved in the MERS-CoV macro domain (Figure 6A). Conservation of catalytically significant residues of ADRP in the MERS-CoV macro domain indicates that the MERS-CoV macro domain may possess ADRP enzymatic activity.

**Structural comparison of macro domains in MERS-CoV, SARS-CoV and HCoV-229E**

The structures of the macro domains of other CoVs pathogenic to humans, including SARS-CoV (36) and HCoV-229E (43), have been determined. Superposition of structures of MERS-CoV, SARS-CoV and HCoV-229E macro domains shows that the major structural divergence lies in the α1 helices, which participates in stabilization of ADP-ribose (Figure 6B). Of note, in terms of the ADP-ribose binding pockets of the three structures, the structures of ADP-ribose appear at different degrees of curvature at the adenine moieties. In the MERS-CoV macro domain, the side chain of Asp20 contacting ADP-ribose points into the cavity that holds adenine moiety. In the SARS-CoV macro domain, the side chain position of the equivalent residue, Asp23, varies significantly from that of Asp20 in the MERS-CoV macro domain. This variation in side chain positions for Asp residues may result from different compositions of amino acids in the α1 helix. In the MERS-CoV macro domain, Asp20 forms two hydrogen bonds with the N6 atom in a pyrimidine ring of ADP-ribose and nitrogen in the Ile22 backbone amide in the α1 helix via the same oxygen atom on its side chain, thereby dragging the Asp20 side chain into the adenine cavity. In contrast, in the SARS-CoV macro domain, Asp23 forms a hydrogen bond with the N6 atom of adenine via one of the oxygen atoms in its side chain and with nitrogen atoms in Val25 and Val26 backbone amides via another. Hydrogen bonding with Val25 and
Val26 of Asp23 in the SARS-CoV macro domain causes a variation in side-chain orientation from that for Asp20 in the MERS-CoV macro domain. Furthermore, in the MERS-CoV macro domain, the lengths of hydrogen bonds formed by the Asp20 side chain with Ile22 and ADP-ribose are 2.96 Å and 2.82 Å, respectively. In the SARS-CoV macro domain, the lengths of hydrogen bonds formed by the Asp23 side chain with Val25 and ADP-ribose are 3.04 Å and 2.87 Å, respectively (Figure 6C). The differential strength of hydrogen bonds formed by Asp with ADP-ribose and residues in the α1 helix of the MERS-CoV and SARS-CoV macro domains may result from the presence of different residues in α helices, given that Ile22 in MERS-CoV appears to be a stronger electron acceptor than Val25 in SARS-CoV, and from variations in side chain orientation of Asp residues in both structures. As compared with Asp20 in MERS-CoV and Asp23 in SARS-CoV, the equivalent residue in HCoV-229E is Asp19, which does not contact ADP-ribose. Instead of forming a hydrogen bond directly with ADP-ribose, the side chain of Asp19 in HCoV-229E contacts Thr22 in the α1 helix via hydrogen bonding with oxygen and nitrogen atoms in the side chain and backbone amides of Thr22, respectively. Hydrogen bonding with Thr22 drags the side chain of Asp19 in the HCoV-229E macro domain away from the adenine cavity as compared with the position of Asp20 in the MERS-CoV macro domain (Figure 6D). Consistent with previous study, the thermodynamic profile (ΔG<0, ΔH<0, and -TΔS<0) of ADP-ribose binding to the HCoV-229E macro domain suggests less contribution of the hydrogen bond to stabilization of ADP-ribose (41) (Table 2). Variations in strength of the hydrogen bond and orientation of the side chain in Asp residues may result in differential binding affinities of ADP-ribose observed in macro domains of MERS-CoV (Kd 2.95 µM), SARS-CoV (Kd 24 µM) (36) and HCoV-229E (Kd 28.9 µM) (41). The relation between binding affinities of ADP-ribose in macro domains and differential pathogenicity of human CoVs needs further investigation.

**Conclusion**

Taken together, our biochemical study shows higher binding affinity for ADP-ribose in the MERS-CoV macro domain than macro domains of CoVs characterized to date. Structural analysis revealed that differences in the context of hydrogen bonds formed by the conserved Asp with ADP-ribose and residues in α helices in macro domains of MERS-CoV, SARS-CoV and HCoV-229E may result in differential binding affinities for ADP-ribose. Our studies provide biochemical basis for further investigating the role of macro domain in MERS-CoV infection and also the precise structural information for the design of novel antiviral drugs.

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FOOTNOTES

Protein data bank accession number

The atomic coordinates and structure factors for MERS-CoV macro domain in complex with ADP-ribose (codes 5DUS) have been deposited in the Protein Data Bank (http://wwpdb.org/)

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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: CHH conceived the study. CCC and MHL performed purification of the enzyme, biochemical assays, DSF, ITC and crystallization. CCC, MHL and CYC collected X-ray data. CCC and CHH determined and analyzed the crystal structure. CCC and CHH contributed to the manuscript writing. All authors reviewed the results and approved the final version of the manuscript.
**FIGURE LEGENDS**

**Figure 1.** Genome organization of Middle East respiratory syndrome coronavirus (MERS-CoV).
Schematic diagram of the composition of structural and non-structural proteins (NSPs) in MERS-CoV genome. Functional domains of NSP3 are highlighted. M<sup>pro</sup>, main (or 3CL) protease; RdRp, RNA-dependent RNA polymerase; Hel, helicase; ExoN, exoribonuclease; NendoU, endoribonuclease; OMT, 2′-O-methyltransferase; S, spike protein; M, membrane protein; N, nucleocapsid protein; MLS, mitochondria localization signal; Macro, macro domain; SUD-M, severe acute respiratory syndrome (SARS)-unique domain-M subdomain; PL<sup>pro</sup>, papain-like protease. NSPs encoded by ORF1a and ORF1b are numbered in green and blue, respectively.

**Figure 2.** Folding of MERS-CoV macro domain.
The CD spectra were recorded at 25°C with 10 μM of MERS-CoV macro domain in CD buffer (20 mM phosphate buffer, pH 3.5-8.5) from 260 to 190 nm.

**Figure 3.** ADP-ribose binding of MERS-CoV macro domain.
(A) Thermal denaturation of MERS-CoV macro domain. CD spectra were recorded at 220 nm with 10 μM MERS-CoV macro domain in CD buffer (20 mM phosphate buffer, pH 7.5) from 25°C to 95°C. The scatterplot shows the MERS-CoV macro domain with and without pre-incubation with 1 mM ADP-ribose, in red and blue, respectively. The melting temperature (Tm) was calculated by using the maximum of the first derivative of the CD signal; the black arrow indicates the shift of Tm. (B) Differential scanning fluorimetry of MERS-CoV macro domain by thermal shift assay on incubation with increasing concentrations of ADP-ribose. Data are mean±SE from 3 independent experiments. Data were fitted by the means of 3 independent experiments. (C) Isothermal titration calorimetry analysis of ADP-ribose binding to MERS-CoV macro domain. Upper panel, raw data in μJ/s versus time showing heat release on injection of 1.14 mM ADP-ribose into a 980-μL cell containing 0.057 mM MERS-CoV macro domain. Lower panel, integration of raw data yielding the heat per mole versus molar ratio. The insert shows thermodynamic parameters of the experiment.

**Figure 4.** Overall structure of MERS-CoV macro domain in complex with ADP-ribose.
(A) Structure of the MERS-CoV macro domain is represented by a ribbon model with helices, strands, and loops in magenta, yellow, and blue, respectively. ADP-ribose is displayed in sticks with carbon in green, oxygen in red, nitrogen in blue, and phosphorus in orange. The 2F<sub>e</sub>-F<sub>c</sub> difference map, contoured at 1σ, was calculated at 1.43-Å resolution from a model with the ligand omitted. (B) Topology diagram of MERS-CoV macro domain with the same colors as with ribbon representation.

**Figure 5.** Detailed view of ADP-ribose binding site in MERS-CoV macro domain.
(A) A close-up of interactions in MERS-CoV macro domain with ADP-ribose binding. Amino acids and ADP-ribose are shown as sticks with carbon in marine blue and yellow, respectively; oxygen in red; nitrogen in blue; and phosphorus in orange. Water molecules are shown as green spheres. Hydrogen bonds are black dashed lines. (B) Interactions between MERS-CoV macro domain and ADP-ribose. Interactions between MERS-CoV macro domain and ADP-ribose were generated by using LigPlot+ (55). ADP-ribose and surrounding residues are shown as ball-and-stick models with carbon in black, nitrogen in blue, oxygen in red, and phosphorus in purple. Atomic bonds in ADP-ribose and the MERS-CoV macro domain are in purple and yellow, respectively. Residues contacting ADP-ribose via hydrogen bonds including Asp20, Ala21, Gly44, Gly46, Ile47, Ser126, Gly128, Ile129, and Phe130 are highlighted in green with hydrogen bonds shown as dashed lines and bond length as numeric numbers. Residues that provide hydrophobic interactions with ADP-ribose including Gly19, Ala36, Asn38, Lys42, His43, Gly45, Ala48, Val52, Pro123, Leu124, Ala127 and Asn154 are in black with red eyelash symbols.

Figure 6. Structural comparison of MERS-CoV, SARS-CoV, and HCoV-229E macro domains.

(A) Structure-based sequence alignment of CoV macro domains. Shown are MERS-CoV (PDB code: 5DUS); SARS-CoV (PDB code, 2FAV); human coronavirus 229E (HCoV-229E; PDB code, 3EWR); HCoV-NL63 (PDB code, 2VRI); and feline CoV (FCoV; PDB code, 3JZT); infectious bronchitis virus (IBV; PDB code, 3EWP). Secondary structures of MERS-CoV macro domain are depicted on the top of the alignment with arrows for β strands and cylinders for α helices. Consensus amino acids among macro domains in CoVs with similarity score > 0.7 are framed in yellow and depicted at the bottom of the alignment. Identical amino acids are in white and framed in red. Blue and green arrowheads on the top indicate amino acids forming hydrogen bonds and providing hydrophobic interactions with ADP-ribose, respectively. Yellow arrowheads at the bottom indicate equivalent amino acids in SARS-CoV macro domain found to abolish or decrease ADRP enzymatic activities when mutated. The number of residues corresponding to MERS-CoV macro domain indicated by blue, green and yellow arrowheads is shown on the top of the alignment. (B) Superposition of macro domains. Structures are shown as a ribbon model with MERS-CoV in blue, SARS-CoV in pink and HCoV-229E in green. ADP-ribose molecules are shown as a stick model. Structural divergence is circled with a black oval. (C) Comparison of interactions in the adenine cavity of MERS-CoV and SARS-CoV macro domains. Amino acids and ADP-ribose are shown as a stick model. Hydrogen bonds are shown as dashed lines and bond lengths are indicated in Å units. (D) Comparison of interactions in adenine cavities of MERS-CoV and HCoV-229E macro domains. Amino acids and ADP-ribose are shown as a stick model. Hydrogen bonds are shown as dashed lines.
Table 1. Data collection and refinement statistics of MERS-CoV macro domain in complex with ADP-ribose.

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<tr>
<td>Wavelength (Å)</td>
<td>0.99347</td>
<td>1.00545</td>
</tr>
<tr>
<td>Resolution Range (Å)</td>
<td>26.51-1.73 (1.79-1.73)</td>
<td>22.53-1.43 (1.48-1.43)</td>
</tr>
<tr>
<td>Unique No. of Reflections</td>
<td>17591</td>
<td>31889</td>
</tr>
<tr>
<td>Total No. of Reflections</td>
<td>229930</td>
<td>186275</td>
</tr>
<tr>
<td>I/σa</td>
<td>43.7 (4.5)</td>
<td>37.2 (6.8)</td>
</tr>
<tr>
<td>Rmerge a,b (%)</td>
<td>6.7 (49.1)</td>
<td>2.9 (25.1)</td>
</tr>
<tr>
<td>Completenessa (%)</td>
<td>99.1 (98.7)</td>
<td>99.8 (100.0)</td>
</tr>
<tr>
<td>Redundancya (%)</td>
<td>13.1 (12.6)</td>
<td>5.8 (5.8)</td>
</tr>
<tr>
<td>CC₁/₂ ad</td>
<td>0.989 (0.953)</td>
<td>0.993 (0.972)</td>
</tr>
<tr>
<td>CC ano e</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Anomalous redundancya</td>
<td>6.9 (6.6)</td>
<td></td>
</tr>
<tr>
<td>Anomalous completenessa (%)</td>
<td>98.9 (98.6)</td>
<td></td>
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<table>
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<tr>
<th>Refinement statistics</th>
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<tr>
<td>Resolution (Å)</td>
<td>1.43</td>
</tr>
<tr>
<td>Rwork (%) / Rfree (%)</td>
<td>12.73 / 16.19</td>
</tr>
<tr>
<td>R.M.S.D.</td>
<td></td>
</tr>
<tr>
<td>Bonds (Å)</td>
<td>0.007</td>
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<tr>
<td>Angles (°)</td>
<td>1.213</td>
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<tr>
<td>Mean B-factor (Å²)</td>
<td>20.6</td>
</tr>
<tr>
<td>Protein</td>
<td>17.2</td>
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<tr>
<td>ADP-ribose</td>
<td>37.6</td>
</tr>
<tr>
<td>Water</td>
<td>36.3</td>
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<td>Ramachandran plot (%)</td>
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<td>Favored</td>
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<tr>
<td>Allowed</td>
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<tr>
<td>Outliers</td>
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Values in parentheses are for the highest resolution shell.

\[ R_{\text{merge}} = \frac{\sum_h \sum_i |I_{h,i} - I_{h}|}{\sum_h \sum_i I_{h,i}}, \]
where \( I_h \) is the mean intensity of the \( i \) observations of symmetry related reflections of \( h \).

\[ R_{\text{work}}/R_{\text{free}} = \frac{\sum |F_{\text{obs}} - F_{\text{calc}}|}{\sum F_{\text{obs}}}, \]
where \( F_{\text{calc}} \) is the calculated protein structure factor from the atomic model (\( R_{\text{free}} \) was calculated with 5% of the reflections selected).

\( CC_{1/2} \) is a percentage of correlation between intensities from random half-datasets (56).

\( CC_{\text{ano}} \) is a percentage of correlation between random half-datasets of anomalous intensity differences.
Table 2. Kinetic parameters for binding of adenosine diphosphate (ADP)-ribose in coronavirus (CoV) macro domains.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Method</th>
<th>Kd</th>
<th>ΔH</th>
<th>ΔS</th>
<th>-TΔS</th>
<th>ΔG</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µM)</td>
<td>(KJ/mol)</td>
<td>(J/mol·K)</td>
<td>(KJ/mol)</td>
<td>(KJ/mol)</td>
<td></td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>ITC</td>
<td>2.95</td>
<td>-91.04</td>
<td>-199.5</td>
<td>59.48</td>
<td>-31.56</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>DSF</td>
<td>3.12</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>ITC</td>
<td>24</td>
<td>-73.39</td>
<td>-153.9</td>
<td>46.65</td>
<td>-26.74</td>
<td>(36)</td>
</tr>
<tr>
<td>HCoV-229E</td>
<td>ITC</td>
<td>28.9</td>
<td>-14.54</td>
<td>38.1</td>
<td>-11.36</td>
<td>-25.9</td>
<td>(41)</td>
</tr>
<tr>
<td>FCoV</td>
<td>Pull-down-based</td>
<td>~400</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>(42)</td>
</tr>
</tbody>
</table>

MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV, severe acute respiratory syndrome CoV; FCoV, feline CoV
Figure 1.
Figure 2.
Figure 4.
Figure 5.
Figure 6.
Macro Domain from Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is an Efficient ADP-ribose Binding Module: Crystal Structure and Biochemical Studies
Chao-Cheng Cho, Meng-Hsuan Lin, Chien-Ying Chuang and Chun-Hua Hsu

*J. Biol. Chem.* *published online January 5, 2016*

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