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Review Article

SARS Virus Papain-Like Protease: A Mysterious Weapon

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ABSTRACT

Introduction: Papain-like protease (PLpro) of SARS-CoV in association with 3Chemotrypsin-like protease (3CLpro or Mpro) are two proteases which auto-proteolyze replicase polyproteins pp1a/pp1ab. These poly-proteins are translated from ORF1a/ORF1b of the virus genome. Cleavage of pp1a/pp1ab releases non-structural proteins of the virus which orchestrate viral replication. In addition, PLpro as a deubiquitinase and deISGylase modifies the proteins involved in recognition of the virus by the sensors of host cell innate immunity system. In this manner, the virus reforms the ubiquitination and ISGylation of the cell proteins to progress its own replication without any interference from host cell restrictive strategies against the viruses. Furthermore, PLpro blocks IRF3 activation independent of deubiquinating processes. Besides, PLpro induces pulmonary fibrosis through pathways involving ROS and MAPK.

Conclusion: Inhibition of PLpro allows innate immunity to sense and react against the invasion of SARS-CoV and to activate IRF3 to induce type I IFN expression. Thenceforth, proper development and signaling of innate immunity result in a long-term efficient cell/humoral adaptive immunity. Moreover, suppression of PLpro prevents cleavage of nsp3 and hence replication of the virus and through abolishing ubiquitin-proteasome/MAPK/ERK- and ROS/MAPK-mediated pathways prevent pulmonary fibrosis.

Abbreviations: 3CLpro: 3Chemotrypsin-like protease; ARDS: acute respiratory distress syndrome; CoV: corona virus; ISG: interferon stimulating gene; IFN: interferon; IRF: interferon regulatory factor; MAPK: mitogen activated protein kinase; Mpro: Main protease; ORF: open reading frame; PLpro: papain-like protease; pp1a/pp1ab: poly-protein 1a/poly-protein 1ab; ROS: reactive oxygen species; STAT: signal transducers and activators of transcription

Introduction:

Coronaviruses (CoVs) are enveloped non-segmented single-stranded large positive RNA viruses with the ability to induce gastrointestinal, neurologic and respiratory diseases in human subjects as well as animals.(1, 2) They are classified in Riboviria realm containing all positive-strand, double-strand or negative-strand genomic RNA viruses which utilize RNA-directed RNA polymerases (RdRPs) in the process of replication. Riboviria realm includes Nidovirales order which is further divided to Coronaviridae, Arteriviridae, and Roniviridae families. Coronaviridae family consists of two subfamilies called Coronavirinae (with the largest RNA genome) and Torovirinae. The four genera of Coronavirinae subfamily include Alpha-coronaviruses (αCoV), Beta-

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coronaviruses (βCoV), Delta-coronaviruses (δCoV), and Gamma-coronaviruses (γCoV).(2-4) Two highly pathogenic human CoVs, Severe Acute Respiratory Syndrome CoV (SARS-CoV) and Middle East Respiratory Syndrome CoV (MERS-CoV) belong to βCoVs.(5) These two CoVs, which are actually zoonotic viruses with the evolution to utilize receptors on host cells or cellular machinery of humans have transmitted from their restricted animal kingdom to human subjects. Their spread resulted in epidemic of SARS and MERS in 2002-2003 and 2012, respectively, with the mortality rate of about 10% in the former and 43% in the latter.(6, 7)

All coronaviruses contain an RNA-type genome which is similar to eukaryotic cell mRNA as it exhibits both 5' caps and 3' poly(A) tails, yet it is much larger than the latter.(8) Replication of these viruses should follow a very delicate pathway. Otherwise, reproducing process of this whole large genome with high probability of mutation in one gene in each round of replication may reach to an error threshold.(9, 10) The whole genome of SARS-CoV comprises some sequences called "open reading frame" (ORF) with the potentials to be translated into proteins.(11) There are two essential ORFs in the 5' two-thirds of SARS-CoV genome: ORF1a and ORF1b. Translation of these ORFs results in the production of replicase polyproteins (pp): pp1a and pp1ab.(12) Autoproteolysis of these polyproteins produces 16 non-structural proteins (nsp1 to nsp16) which provide four key enzymes participating in replication of SARS-CoV: RNA-dependent RNA polymerase (RdRp), 3Chemotrypsin-like proteinase (3CLpro or Mpro), a papain-like proteinase (PLpro) and a helicase. Intriguingly, PLpro and 3CLpro are included in nsp3 and nsp5 molecules, respectively.(13,14) Among these essential functional proteins, PLpro plays a critical role not only in the proteolytic cleavage of nsp1/nsp2, nsp2/nsp3 and nsp3/nsp4 from pp1a but also allows the virions to evade the intracellular sensors of innate immunity.(15)

Nsp3 structure and function

Nsp3 of SARS-CoV is the largest protein (215KDa) cleaved from pp1a/1ab by PLpro which is itself amazingly a part of nsp3, as well. Apart from its proteolytic function, nsp3 participates in processing of viral RNA. Essential for replication-transcription complex (RTC) formation in co-expression with nsp4 and nsp6, nsp3 is needed for anchoring of RTC to double-membrane vesicles (DMVs) and convoluted membranes (CMs) originating from endoplasmic reticulum of the host cells; it acts as a scaffolding protein to ease interactions with and of other proteins involved in the replication/transcription process of the virus.(16-19) Nsp3 in SARS-CoV is composed of several domains such as two ubiquitin-like (UBl) domains, the N-terminal Glu-rich acidic-domain, the papain-like protease domain, a papain-like noncanonical domain (PLnc), a SARS unique domain, multiple transmembrane domains and an adenine dinucleotide phosphate ribose-1 phosphatase (ADRP) domain. The latter is called macrodomain with the ability to attach to mono and poly(ADP-ribose) (MAR-PAR), deacetylate O-acetyl-ADP-ribose, and dephosphorylate ADP-ribose-1"-phosphate (Appr-1"-p). Some studies showed that macrodomain could detach MAR from ADPribosylated proteins (deMARylating) in mammals. Moreover, macrodomain gives the coronaviruses the potential to evade the innate immune system.(10, 19-21)

The crystal structure of PLpro proteins and UBls in Riboviria realm can be found in (Tables 1-3).

Papain-like protease (PLpro)

PLpro incorporated in the molecule of nsp3 plays its critical role in cleavage of nsp1/2, nsp2/3 and nsp3/4. Without cleavage processing of replicase pp1a/pp1ab, nsps are unable to do their proper jobs and assemble in RTC formation.(17, 21)

Apart from having a triad of cysteine-histidine-aspartic acid content, PLpro encompasses a zinc binding motif. Being conserved in PLpro of SARS-CoV, the presence of aspartic acid in catalytic triad and the settlement of four cysteine residues in zinc binding motif are mandatory for PLpro to be functional.(22,23) There exists a ubiquitine-like (UBl2) domain in PLpro amino-terminal.(23) Despite the presence of this UBl2, a zinc motif and its open cleavage site, PLpro was found to have a deubiquitinating and de-ISGylating role.(24) It is worth declaring that PLpro in combination with UBl2 seems rather to be an ordered protein as it shows less than 10% changes in conformational shape prevailing in the region of UBl2 connection in 100ns MD simulation (Figure 1).(25) However, the number of hotspots in the structure of PLpro plus and without UBl2 found through Molegro Virtual Docker (MVD) algorithm has been uncovered to be more than the single site of the triad of cysteine-histidine-aspartic acid. These hotspots might represent the cavities of active sites of this protease. The significance of these cavities in this rather ordered protein should be more studied. Moreover, MVD algorithm has shown that dimerization of PLpro might expand the hotspots of this deubiquitinase (Figure 2).

Table 1. UBL1 structures based on its amino acid sequence in crystal structures of severe acute respiratory syndrome-related coronavirus of Riboviria Kingdom

	PDB ID	Organism	Realm	Released
1	2IDY: A	Severe acute respiratory syndrome-related coronavirus	Riboviria	2006-12-05
2	2GRI: A	Severe acute respiratory syndrome-related coronavirus	Riboviria	2006-12-19
3	2M0A: A	Murine hepatitis virus strain A59	Riboviria	2013-01-23

*Table 2. Papain-Like Structures Based on its amino acid Sequence in Crystal Structures of **Severe Acute Respiratory Syndrome**-Related Coronavirus of Riboviria Kingdom*

	PDB ID	Method & Resolution	Realm	Released
1	2FE8:A,B,C	X-RAY DIFFRACTION 1.85 Å	Riboviria	2006-03-21
2	3E9S:A	X-RAY DIFFRACTION 2.5 Å	Riboviria	2008-10-07
3	3MJ5: A,B	X-RAY DIFFRACTION 2.63 Å	Riboviria	2010-06-30
4	4M0W: A	X-RAY DIFFRACTION 1.4 Å	Riboviria	2014-02-12
5	4MM3: B	X-RAY DIFFRACTION 2.752 Å	Riboviria	2014-07-02
6	5E6J: A,D	X-RAY DIFFRACTION 2.85 Å	Riboviria	2016-05-18
7	5TL6: B,D	X-RAY DIFFRACTION 2.618 Å	Riboviria	2017-05-03
8	5TL7: B,D	X-RAY DIFFRACTION 2.44 Å	Riboviria	2017-05-03
9	5Y3E: A	X-RAY DIFFRACTION 1.65 Å	Riboviria	2018-01-10
10	5Y3Q: A	X-RAY DIFFRACTION 1.65 Å	Riboviria	2018-01-10

Table 3. Papain-Like Crystal Structures of Related Viruses

	PDB ID	Organism	Resolution	Released
1	4OVZ: A, B	SARS coronavirus Urbani	2.50 Å	2014-04-23
2	4OW0: A, B	SARS coronavirus Urbani	2.10 Å	2014-04-23
3	4P16: A	Human betacoronavirus 2c EMC/2012	2.50 Å	2014-05-07
4	4PT5: A	Human betacoronavirus 2c EMC/2012	2.59 Å	2014-05-21
6	4REZ: A	Human betacoronavirus 2c Jordan-N3/2012	2.80 Å	2014-10-22
7	4RF0: A	Human betacoronavirus 2c Jordan-N3/2012	2.80 Å	2014-10-22
8	4RF1: A	Human betacoronavirus 2c Jordan-N3/2012	2.15 Å	2014-10-22
9	4WUR: A	Betacoronavirus England 1	3.16 Å	2014-11-26
10	4X2Z: A	Avian infectious bronchitis virus (strain Beaudette)	2.15 Å	2015-01-28
11	4RNA: A	Human betacoronavirus 2c EMC/2012	1.79 Å	2015-03-25
12	4R3D: A.B	Betacoronavirus England 1	2.81 Å	2015-08-19
13	4YPT: A	Murine hepatitis virus strain A59	2.60 Å	2015-08-26
14	5KO3: A	Middle East respiratory syndrome-related coronavirus	1.94 Å	2017-01-25
15	5V6A: A	Human betacoronavirus 2c EMC/2012	2.70 Å	2017-05-10
16	5V69	Human betacoronavirus 2c EMC/2012	2.55 Å	2017-05-10
17	5W8U: A,C	Human betacoronavirus 2c EMC/2012	2.41 Å	2017-09-27
18	5W8T: A, C	Human betacoronavirus 2c EMC/2012	2.75 Å	2017-09-27
19	5WFI: A.B	Murine hepatitis virus strain A59	1.85 Å	2018-08-01
20	6BI8: A,B	Human betacoronavirus 2c EMC/2012	2.29 Å	2018-11-07

Ubiquitination and ISGylation

Ubiquitins (UBs) are small globular proteins of 76 amino acids. These conserved proteins with the ability to covalently bind to and modify other proteins, play a critical part in host cell restriction of viral replication. Deubiquiting enzymes (DUBs) hydrolyze isopeptide bonds between UBs and their substrates.(26) Ubiquitination and deubiquitination are highly time-, compartment- and substrate quality-dependent processes contributing to both degradation and activation of functional proteins and enzymes to confront intracellular dyshomeostasis as fast as possible.(27) Different cellular functions may be anticipated to occur depending on the lysine residues (K6, K11, K27, K29, K33, K48, K63) which participate in conjugation of proteins with UB or poly-UB chains. K48 poly-UB linkage leads to degradation of the target proteins with proteasomes while K63 poly-UB conjugation leads to proteasome-independent signals for endocytosis and potentiates antiviral immune signaling pathways like NF-kB as well as DNA repair.(28-30) Ubiquitination is more complicated than being described as signaling responses based on a single type of UB or its sort of conjugation. Ubiquitination may occur as mono or poly, homogenous or heterogenous and linear or branched chain types. A significant amount of K63 UB chains are actually of branched type with a heterogenous linkage of K48-K63. This branched heterogenous chain has been revealed to contribute more to proteasomal protein degradation. Besides, ubiquitination may occur through ester-based linkage with serine and threonine and thiol-ester bonds with cysteine residues in the substrates. (27)

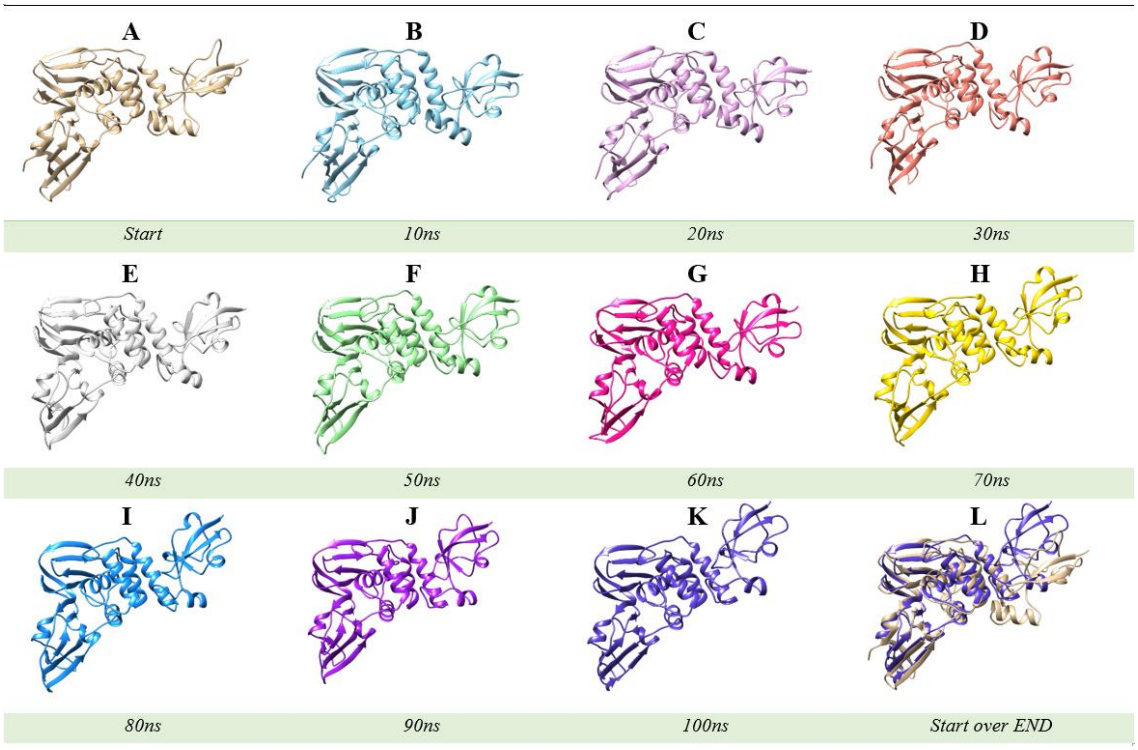

Figure 1. Changes in conformational shape of PLpro in covalent bond with UBl2 in MD-simulation for 100ns (A-K). L shows superimposition of PLpro+UBl2 of A and K. It is obvious that the molecule has changed after 100ns prevailing in the region of UBl2

Invasion of pathogens elicits activation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), NOD-like receptors (NLRs), C type lectin receptors (CLRs) and cytosolic DNA sensors such as members of the Absent in Melanoma2 (AIM2) family. PRRs trigger expression of interferon and cytokines as well as maturation of IL-1β and IL-18 and inducing of adaptive immune responses.(28, 31) Signaling pathways downstream of PRR activation guarantee translocation of transcription factors like interferon regulatory factor 3 (IRF3) and IRF7 to the nucleus and subsequent production of type I interferon (IFN I) which by itself induces signal transducers and activators of transcription 1 (STAT1), STAT2 and IRF9 to form interferon stimulating gene factor3 (ISGF3). The latter upregulates expression of ISG, a ubiquitin like protein with C-terminal LRLRGG sequence and homology to ubiquitin linear dimer.(28, 32) ISGylation (linking of ISG to target proteins) is involved not only in immunity and cellular defense but also in stress responses, cell structure, and motility, or carbohydrate metabolism.(32) ISG prevents virus replication and distribution.(33) Modification of IFNs and other mediators like IRFs and RIG-1 by ubiquitin and ubiquitin-like proteins is required for innate immunity activation.

Deubiquitinating property of PLpro, which is almost 180 times more potent than that of its hydrolyzing property, enables SARS-CoV to destabilize the ubiquitination machinery of the host cell in order to optimize the intracellular environment for its replication. PLpro shows close homology to deubiquitinases (DUBs) such as ubiquitin C terminal hydrolase (UCH-L1), ubiquitin-specific protease 14 (USP14), and herpes-associated ubiquitin-specific protease (HAUSP, also known as USP 7) in the host cells. (22, 34, 35) Furthermore, as C-terminal amino

acid sequence of PLpro processing site contains LXGG residues which is in close similarity to LRLRGG found in UBs, it seems that as a deubiquitinase, PLpro cleaves nsps in their N-terminus.(24, 36) An in vitro study elucidated that PLpro debranches K48 polyUBs more preferably than K63 polyUBs. Amazingly, PLpro has been considered to have the ability to deconjugate ISG from the latter's substrates more efficiently than that of its deubiquinating capability.(24)

Apart from deubiquitinating and deISGylating characteristics of PLpro which dampen the innate immunity response, it has also been demonstrated that PLpro blocks IRF3 phosphorylation, dimerization and nuclear transport. Moreover, deubiquitinating potential of PLpro has been shown to inhibit IRF3 ability to promote IFNβ gene even after this regulatory factor is phosphorylated, dimerized, imported to the nucleus and linked to IFN inducer region of the host genome. PLpro also interacts with stimulator of IFN genes (STING) and prevents the cells from recognizing SARS-CoV RNA and expression of IFN-β.(37)

An in vitro study showed that the presence of UBl bound with PLpro is necessary for its anti-IFN function.(38) Intriguingly, it has been uncovered that the potency of PLpro function among members of SARS-CoV determines the virulence trait of these viruses.(39)

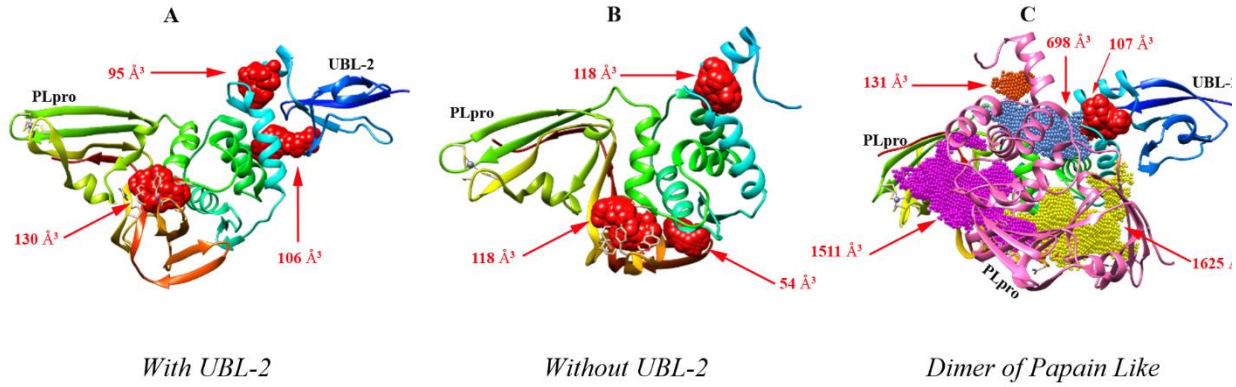

Figure 2. All MVD-detected cavities and their calculated volumes (in Å³) in Papain-Like protease (PDB ID: 3mj5); detected cavities are represented in red. A: PLpro with UBl2; B: PLpro without UBl2; C: dimerization of PLpro (40)

Fibrotic effects of PLpro:

Twenty percent of patients recovered from SARS-induced ARDS was demonstrated to be suffering from pulmonary fibrosis 9 months after discharging home. Early phase of infection with SARS-CoV is associated with upregulation of a pro-fibrotic factor, TGF-β, in plasma and lungs.(41) PLpro of SARS-CoV increases the deposition of type I collagen in the lungs. This protease in the host cells induces production of TGF-β via ubiquitin-proteasome/p38MAPK/(ERK1/2)-mediated signaling as well as through activating ROS-mediated p38MAPK/STAT3 pathway which promotes expression of early growth response 1 (Egr-1) with the ability to bind TGF-β promoter region.(41, 42)

Conclusion:

Deubiquinating and deISGylating potential of PLpro provides the SARS-CoV with the ability to evade the innate immunity. Consequently, innate immunity may not be activated and present efficiently the antigenic epitopes of SARS-CoV to naïve T cells to induce an adaptive immunity response in a short time after the cells are infected. PLpro is also the determinant of virulence among SARS-CoVs. In addition, contribution of PLpro to fibrosis of the lungs makes this protease a weapon against the patients who survive.

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