In Silico Analysis of ORF1ab in Coronavirus HKU1 Genome Reveals a Unique Putative Cleavage Site of Coronavirus HKU1 3C-Like Protease

Patrick C.Y. Woo1,2,3,†, Yi Huang1,†, Susanna K.P. Lau1,2,3, Hoi-wah Tsoi1, and Kwok-yung Yuen1*,1,2,3

1Department of Microbiology, The University of Hong Kong, University Pathology Building, Queen Mary Hospital, Hong Kong, 2Research Centre of Infection and Immunology, Faculty of Medicine, The University of Hong Kong, and 3State Key Laboratory of Emerging Infectious Diseases (The University of Hong Kong), Hong Kong

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Abstract: Recently we have described the discovery and complete genome sequence of a novel coronavirus associated with pneumonia, coronavirus HKU1 (CoV-HKU1). In this study, a detailed in silico analysis of the ORF1ab, encoding the 7,182-amino acid replicase polyprotein in the CoV-HKU1 genome showed that the replicase polyprotein of CoV-HKU1 is cleaved by its papain-like proteases and 3C-like protease (3CLpro) into 16 polypeptides homologous to the corresponding polypeptides in other coronaviruses. Surprisingly, analysis of the putative cleavage sites of the 3CLpro revealed a unique putative cleavage site. In all known coronaviruses, the P1 positions at the cleavage sites of the 3CLpro are occupied by glutamine. This is also observed in CoV-HKU1, except for one site at the junction between nsp10 (helicase) and nsp11 (member of exonuclease family), where the P1 position is occupied by histidine. This amino acid substitution is due to a single nucleotide mutation in the CoV-HKU1 genome, CAG/A to CAT. This probably represents a novel cleavage site because the same mutation was consistently observed in CoV-HKU1 sequences from multiple specimens of different patients; the P2 and P1′-P12′ positions of this cleavage site are consistent between CoV-HKU1 and other coronaviruses; and as the helicase is one of the most conserved proteins in coronaviruses, cleavage between nsp10 and nsp11 should be an essential step for the generation of the mature functional helicase. Experiments, including purification and C-terminal amino acid sequencing of the CoV-HKU1 helicase and trans-cleavage assays of the CoV-HKU1 3CLpro will confirm the presence of this novel cleavage site.

Key words: Coronavirus HKU1, ORF1ab, 3C-like protease

Coronaviruses, a genus of the family Coronaviridae, are large, enveloped, positive-stranded RNA viruses that cause respiratory, enteric, hepatic, and neurological diseases in humans and domestic animals. In humans, it has been estimated that coronaviruses [human coronaviruses 229E (HCoV-229E) and OC43 (HCoV-OC43)] are responsible for causing about 5–30% of respiratory tract infections. In late 2002 and 2003, a novel clinical syndrome, Severe Acute Respiratory Syndrome (SARS), affected 30 countries on five continents with more than 8,000 cases and 750 deaths. A novel coronavirus, the SARS coronavirus (SARS-CoV), has been confirmed to be the aetiological agent, and its genome has been completely sequenced (13, 28, 36, 37, 39, 40).
Materials and Methods

Viral sequences. The predicted amino acid sequences of ORF1ab in CoV-HKU1 were extracted from the CoV-HKU1 genome sequence (GenBank accession no. NC_006577) (48). The corresponding amino acid sequences of ORF1ab in other coronaviruses were extracted from GenBank (HCoV-OC43, GenBank accession no. NC_006577) (48). The amino acid sequences of ORF1ab in CoV-HKU1 were extracted from the CoV-HKU1 genome sequence (GenBank accession no. NC_006577) (48). The corresponding amino acid sequences of ORF1ab in other coronaviruses were extracted from GenBank (HCoV-OC43, GenBank accession no. NC_006577) (48). The amino acid sequences of ORF1ab in CoV-HKU1 were extracted from the CoV-HKU1 genome sequence (GenBank accession no. NC_006577) (48).

In silico analysis. Multiple alignment was performed using ClustalX 1.83 (46). Protein family analysis was performed using PFAM (2). Secondary structure prediction was performed using PROFsec (38). Three-dimensional modeling of 3CL\(^\text{pro}\) of CoV-HKU1 was performed using 3CL\(^\text{pro}\) of SARS-CoV as the template (54). Manually corrected sequence alignment and homology modeling requirement were submitted via Deep View – spdbv 3.7 (14) to Swiss-model Protein Modeling web server (http://swissmodel.expasy.org/) (40). The 3D structure was displayed using MOLMOL 2K.2 (22). Phylogenetic tree construction was performed using neighbour joining method with ClustalX 1.83.

Results and Discussion

General Overview

The replicase polyprotein (7,182 amino acids) of CoV-HKU1 is translated from ORF1a (13,395 nt) and ORF1b (8,154 nt). Similar to other coronaviruses, a slippery sequence (UUUAAC), followed by sequences that form a putative pseudoknot structure, are present (Fig. 1) (5, 6). Translation will presumably occur by a -1 RNA-mediated ribosomal frameshift at the end of the slippery sequence. Therefore, instead of reading the transcript as UUUAACGGGG, it will be read as UUUAACCGGG. In infectious bronchitis virus (IBV) and SARS-CoV, site directed mutagenesis experiments have confirmed the importance of the slippery sequence as well as the pseudoknot structure for the frameshift to occur (7, 45).

Proteolytic Cleavage of Replicase Polyprotein

Multiple alignments between the replicase polyprotein of CoV-HKU1 and those of other coronaviruses reveal the orthologs of two types of proteases, papain-like proteases (PL\(^\text{pro}\)) and 3CL\(^\text{pro}\). As in other coronaviruses and by multiple alignment and analysis of sequences around consensus cleavage sites, the replicase polyprotein of CoV-HKU1 is cleaved by its PL\(^\text{pro}\) and 3CL\(^\text{pro}\) into 16 polypeptides (Table 1). The three N-terminal cleavage sites are putatively cleaved by the PL\(^\text{pro}\), whereas the others are putatively cleaved by the 3CL\(^\text{pro}\).

The putative PL\(^\text{pro}\) cleavage sites in the replicase polyprotein of CoV-HKU1 follow the general rules determined by site directed mutagenesis experiments in other group 2 coronaviruses. In MHV, site directed mutagenesis studies have shown that at the p28/p65 cleavage site, a basic residue (arginine or lysine) in the
P5 position, arginine in the P2 position, and glycine in the P1 position are the critical amino acids for PL1 pro recognition and processing (17). In CoV-HKU1, arginine, arginine, and glycine are present in these three positions respectively (Table 2). In MHV, site-directed mutagenesis studies have shown that at the p65/nsp1 cleavage site, arginine in the P5 position, alanine in the P1 position, and glycine in the P1' position are the critical amino acids for PL1 pro recognition and processing (4). In CoV-HKU1, arginine, alanine and glycine are present in these three positions respectively (Table 2).

In contrast to the putative PL1 pro cleavage sites, analysis of the putative cleavage sites of the 3CLpro in the replicase polyprotein of CoV-HKU1 revealed a unique putative cleavage site by the 3CLpro of CoV-HKU1. The 3CLpro of coronaviruses are named as such because their structures and substrate specificities resemble those of the 3C proteases in picornaviruses. In all known coronaviruses, the P1 positions at the cleavage sites of the 3CLpro are occupied by glutamine, whereas the P1' positions are occupied by aliphatic amino acids, including alanine, glycine, cysteine, asparagine and serine (3, 9).

Table 1. Characteristics of the 16 putative polypeptides derived from replicase polyprotein in CoV-HKU1

| Polypeptides | First amino acid | Number of amino acid residues | Characteristics |
|--------------|-----------------|-------------------------------|-----------------|
| p28          | Met$^{22}$-Gly$^{222}$ | 222                           | Unknown         |
| p65          | Ile$^{223}$-Ala$^{89}$   | 587                           | Unknown         |
| nsp1         | Gly$^{401}$-Gly$^{738}$ | 2,029                          | ATR, AC, ADRP, PL1 pro, PL2 pro, Y domain |
| p44          | Val$^{239}$-Gln$^{554}$ | 496                           | Hydrophobic domain |
| nsp2         | Ser$^{335}$-Gln$^{637}$ | 303                           | 3CLpro           |
| nsp3         | Ser$^{368}$-Gln$^{924}$ | 287                           | Hydrophobic domain |
| nsp4         | Ser$^{592}$-Gln$^{915}$ | 92                            | Unknown          |
| nsp5         | Ala$^{404}$-Gln$^{620}$ | 194                           | Unknown          |
| nsp6         | Asn$^{421}$-Gln$^{520}$ | 110                           | Unknown          |
| nsp7         | Ala$^{432}$-Gln$^{597}$ | 137                           | Unknown          |
| nsp8         | Ser$^{449}$-Val$^{571}$ | 14                            | Unknown (short peptide at the end of ORF1a) |
| nsp9         | Ser$^{498}$-Gln$^{588}$ | 928                           | Pol             |
| nsp10        | Ser$^{536}$-His$^{608}$ | 603                           | Hel             |
| nsp11        | Cys$^{595}$-Gln$^{699}$ | 521                           | ExoN            |
| nsp12        | Ser$^{852}$-Gln$^{903}$ | 374                           | XendoU          |
| nsp13        | Ala$^{884}$-Cys$^{1002}$ | 299                           | 2'-O-MT         |

ATR, acidic tandem repeats; AC, acidic domain; ADRP, ADP-ribosyl 1'-phosphatase; PL1 pro, papain-like protease 1; PL2 pro, papain-like protease 2; 3CLpro, 3C-like protease; Pol, RNA-dependent RNA polymerase; Hel, helicase; ExoN, 3'-to-5' exonuclease; XendoU, poly(U)-specific endoribonuclease and 2'-O-MT, S-adenosylmethionine-dependent 2'-O-ribose methyltransferase.
Table 2. Analysis of amino acid residues around putative cleavage sites of PL<sup>+</sup> and 3CL<sup>+</sup> in replicate polyprotein of CoV-HKU1

| Putative polypeptides | Amino acid residues around putative cleavage sites | MHV, BCoV, HCoV-OC43 (P1<sup>+</sup>) | Putatively cleaved by |
|-----------------------|--------------------------------------------------|--------------------------------------|-----------------------|
|                       | Positions (P1P1<sup>+</sup>)                     | 6 5 4 3 2 1 1' 2' 3' 4' 5' 6'        | P1<sup>+</sup>         |
| p28/p65               | 222|223 L R Q Y R G I K P V L F                 | G/V                   |
| p65/insp1             | 809|810 W R F P C A G R K V N F                | —                     |
| nsp1/p4               | 283|283 F S L K G G G V V L S N L             | G/A                   |
|                       | 333|4335 S T S F L Q S G I V K M              | —                     |
|                       | 363|7|3638 A G V K L Q S K T K R F              | —                     |
|                       | 392|4|3925 E V S Q I Q S K L T D V              | —                     |
|                       | 401|3|4014 D S T V L Q A L Q S E F              | —                     |
|                       | 421|0|4211 A N A V M Q N N E L M P              | —                     |
|                       | 432|0|4321 T S I R L Q A G V A T E              | 3CL<sup>+</sup>        |
|                       | 445|7|4458 S S V A V Q S K D L N F              | —                     |
|                       | 538|5|5386 K S A V M Q S V G A C V              | —                     |
|                       | 598|8|5989 T L P R L H C T T N L F              | Q/C                   |
|                       | 650|9|6510 T F T T L Q S L E N V I              | —                     |
|                       | 688|3|6884 F Y P K M Q A T N D W K              | —                     |

* P1<sup>+</sup> amino acid residues in CoV-HKU1 are the same as those in MHV, BCoV and HCoV-OC43.

The amino acid residues critical for cleavage by PL<sup>+</sup> in MHV as determined by site directed mutagenesis studies are highlighted.

Fig. 2. Multiple alignment of the amino acid sequences around the putative cleavage site between nsp10 and nsp11 in CoV-HKU1, MHV, BCoV and HCoV-OC43. The conserved amino acids from positions P2 to P12' in the four coronaviruses are highlighted.

In most cases, the P1' positions are occupied by alanine, glycine or serine. Occasionally, in group 2 coronaviruses, the P1' positions are occupied by cysteine (30); and in rhinoviruses, by asparagines (3). In CoV-HKU1, multiple alignment with the genome sequences of other coronaviruses revealed that all, except one, P1 positions of putative cleavage sites of 3CL<sup>+</sup> are occupied by glutamine. The exception lies at the junction between nsp10 (helicase) and nsp11 (member of exonuclease family), where the P1 position is occupied by histidine. This probably represents a novel cleavage site due to the following reasons. First, the sequence is genuine instead of a result of RT-PCR or sequencing errors because the same position has been amplified and sequenced four times in different clinical specimens of the index patient (48). Second, the same mutation was consistently found in the genome sequence of the CoV-HKU1 in the second patient with pneumonia (data not shown). Therefore, it is unlikely that it is due to an occasionally occurring mutation in just one patient. Third, in all other group 2 coronaviruses with genome sequences available (MHV, BCoV and HCoV-OC43), the P2, P1', P2', P3', P4', P5', P6', P7', P8', P9', P10', P11' and P12' positions of this cleavage site are all occupied by leucine/valine [shown to be important for 3CL<sup>+</sup> cleavage (35)], cysteine, serine/threonine, threonine, asparagines, leucine, phenylalanine, lysine, aspartic acid, cysteine, serine, lysine/arginine and serine, respectively, which are also present at the corresponding positions in CoV-HKU1.
(Fig. 2). Fourth, this amino acid substitution is probably due to a single nucleotide mutation in the CoV-HKU1 genome, CAG (as in MHV) or CAA (as in BCoV and HCoV-OC43) (which encodes glutamine) to CAT (which encodes histidine). Finally, as the helicase is one of the most conserved proteins in coronaviruses, cleavage between nsp10 and nsp11 should be an essential step for the generation of the mature functional helicase. The present novel putative cleavage site, as well as some other atypical cleavage sites by 3CLpro, were not predicted by a program for prediction of 3CLpro cleavage sites (21). Experiments, including purification of the CoV-HKU1 helicase in its native form and C-terminal amino acid sequencing and trans-cleavage assays of the CoV-HKU1 3CLpro will confirm the presence of this novel cleavage site.

**Putative Polypeptides of the CoV-HKU1 Replicase Polyprotein**

The replicase polyprotein of CoV-HKU1 is putatively cleaved by its PLpro and 3CLpro into 16 polypeptides (Table 1). Among these 16 putative polypeptides, p28, p65, nsp3, nsp4, nsp5, nsp6, nsp7 and nsp8 have no homologues with known functions by BLAST search. Similar to other coronaviruses, the N terminal of the putative nsp1 consists of an acidic domain (amino acids 1–333 of nsp1). However, unlike other coronaviruses, there are 16 tandem copies of a 10-amino acid repeat near the C terminal of the acidic domain. The first 14 tandem copies are perfect repeats of NDDENVVTGD, with four of the 10 amino acids being either glutamic acid or aspartic acid. The last two copies are imperfect repeats of NNDEEIVTGD and NDDQIVVTGD respectively. In picornaviruses, it has been found that there were three tandem copies of 24 imperfect amino acid repeats, named VPg1, VPg2 and VPg3 (10). However, no specific functions have been found for these tandem repeats. Downstream to the acidic domain is the PL1pro (amino acids 334–549 of nsp1), with the characteristic catalytic dyad of cysteine and a downstream histidine. Moreover, similar to the PL1pro of other coronaviruses, four conserved cysteine residues (Cys620, Cys623, Cys635 and Cys647) that may contain a Zn2+ binding motif are present. Downstream to PL1pro is the X domain (amino acids 550–709 of nsp1) that contains putative ADP-ribose 1′,2′-cyclic phosphate (Appr>p) activity [amino acids 575–685 of nsp1 belonging to Appr-1-p processing enzyme family (Pfam accession no. PF01661)]. The putative ADPR activity was first described for this domain by Snijder et al. (43). In other microorganisms, such as *Saccharomyces cerevisiae* and other eukaryotes, ADRP and its functionally related enzyme cyclic nucleotide phosphodiesterase (CPDase), were important for tRNA processing (33). ADP-ribose 1′,2′-cyclic phosphate (Appr>p) is produced as a result of RNA splicing. Appr>p is converted to ADP-ribose 1′-phosphate (Appr-1′p) by CPDase. Appr-1′p is then further processed by ADRP. In other group 2 coronaviruses, both putative ADRP and CPDase (in NS2a) homologues are present (43). Interestingly, in CoV-HKU1 and also SARS-CoV, only ADRP, but not CPDase [NS2a is not present in CoV-HKU1 (48)], is present. Downstream to the X domain, there is a segment of 134 amino acids, also present in other coronaviruses, with no matches of known functions found by BLAST search. Downstream to this segment of unknown function is the putative PL2pro (amino acids 844–1140 of nsp1), with the characteristic catalytic dyad of cysteine and a downstream histidine. Similar to the PL2pro of other coronaviruses, four conserved cysteine residues (Cys626, Cys628, Cys630 and Cys636) that may contain a Zn2+ binding motif are also present. At the C terminal of nsp1, similar to other coronaviruses, there is a Y domain with two hydrophobic stretches and 11 conserved Cys/His residues present in the N terminal 889 amino acids (30). Ziebuhr et al. speculated that this Y domain may be responsible for anchoring nsp1 into membranes and binding cations (55). Downstream to nsp1, p44 (496 amino acid residues), putatively cleaved from nsp1 by PL2pro, with a hydrophobic transmembrane domain, is present.

Three-dimensional structure modeling of the putative 3CLpro (nsp2) of CoV-HKU1 using the three-dimensional structure of SARS-CoV as the template revealed a similar structure for the monomer of the CoV-HKU1 3CLpro as compared to the 3CLpro of SARS-CoV and HCoV-229E (Fig. 3). Three domains were present, domain I (8–101 amino acid residues), domain II (102–184 amino acid residues) and domain III (201–303 amino acid residues). Domains I and II consist of six strands of antiparallel β barrels and domain III is a globular structure with five α helices. The catalytic dyad of the protease is located in a cleft between domains I (His41) and II (Cys165).

As in other coronaviruses, the putative Pol (nsp9) of CoV-HKU1 consists of the N-terminal domain, the fingers subdomain, the palm subdomain and the thumb subdomain (Fig. 4). The N-terminal domain (residues 1–371) of the Pol of CoV-HKU1 shares 52–87% amino acid identities with those of other coronaviruses but <31% amino acid identities with those of other RNA viruses. The function of the N-terminal domain is unclear. On the other hand, the fingers (motifs F and G), palm (motifs A–E) and thumb subdomains of the Pol of CoV-HKU1 are homologous to those of other coronaviruses and some positive-stranded RNA virus-
es, such as hepatitis C virus (of family Flaviviridae) (1), rabbit hemorrhagic disease virus (of family Caliciviridae) (34) and poliovirus 1 (of family Picornaviridae) (15) (Fig. 4). The critical amino acids, aspartic acids in motif A and XDD sequence in motif C, which have been demonstrated to be essential for the function of Pol in other coronaviruses and positive-stranded RNA viruses, are also present in Pol of CoV-HKU1.

Similar to the helicase of SARS-CoV, the putative helicase (nsp10) of CoV-HKU1, which unwinds duplex RNA by nucleoside triphosphate hydrolysis activity, possesses six conserved motifs (18) (Fig. 5). Similar to other coronaviruses, based on the presence of conserved motifs, the putative helicase of CoV-HKU1 should belong to superfamily 1 of the three superfamilies of RNA helicases (12, 19). Furthermore, all nine cysteine residues and three histidine residues that are conserved in the N termini of the helicases in nidoviruses are present in the putative helicase of CoV-HKU1 (Fig. 5). It has been suggested that this cysteine/histidine region constitutes a Zn$^{2+}$-binding domain, which controls the activities of the catalytic domain (42). Similar to the helicases of other nidoviruses and most other helicases of superfamily 1, the helicase of CoV-HKU1 probably unwinds the RNA duplex in a 5' to 3' fashion (41, 44).

The putative nsp11 possesses a putative 3'-to-5' exonuclease (ExoN) domain of the DEDD superfamily (56). The putative nsp12 possesses a putative poly(U)-specific endoribonuclease (XendoU) domain (27).
Fig. 5. Multiple alignment of amino acid sequences of the helicases of CoV-HKU1, MHV, BCoV, HCoV-OC43, SARS-CoV, HCoV-229E and IBV. The nine cysteine residues and three histidine residues that are conserved in the N-termini of the helicases in nidoviruses are highlighted. The six conserved motifs are also depicted.
putative nsp13 possesses a putative S-adenosylmethionine-dependent ribose 2'-O-methyltransferase (2'-O-MT) domain of the RMj family (8). These three putative enzymes, as well as ADRP and CPDase, are enzymes in RNA processing pathways. ExoN, XendoU and 2'-O-MT are enzymes in a small nucleolar RNA processing and utilization pathway, in contrast to the pre-tRNA splicing pathway that ADRP and CPDase belong to. At the moment, no experiments have been performed to confirm the activities of these putative RNA processing enzymes. Further studies are required to elucidate the exact roles of these putative viral enzymes in the corresponding viruses.

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