Characterization of trans- and cis-cleavage activity of the SARS coronavirus 3CL\textsuperscript{pro} protease: basis for the in vitro screening of anti-SARS drugs

Cheng-Wen Lin\textsuperscript{a,b,*}, Chang-Hai Tsai\textsuperscript{c,*}, Fuu-Jen Tsai\textsuperscript{c}, Pei-Jer Chen\textsuperscript{d}, Chien-Chen Lai\textsuperscript{c}, Lei Wan\textsuperscript{c}, Hua-Hao Chiu\textsuperscript{a}, Kuan-Hsun Lin\textsuperscript{a}

\textsuperscript{a}Department of Medical Laboratory Science and Biotechnology, China Medical University, No. 91, Hsueh-Shih Road, Taichung 404, Taiwan, ROC
\textsuperscript{b}Clinical Virology Laboratory, Department of Laboratory Medicine, China Medical University Hospital, Taichung 404, Taiwan, ROC
\textsuperscript{c}Department of Medical Genetics and Medical Research, China Medical University Hospital, Taichung 404, Taiwan, ROC
\textsuperscript{d}Department of Internal Medicine, National Taiwan University College of Medicine, National Taiwan University Hospital, Taipei 100, Taiwan, ROC

Received 6 July 2004; revised 26 July 2004; accepted 12 August 2004

Published online 21 August 2004

Edited by Valdimir Skulachev

Abstract Severe acute respiratory syndrome (SARS) has been globally reported. A novel coronavirus (CoV), SARS-CoV, was identified as the etiological agent of the disease. SARS-CoV 3C-like protease (3CL\textsuperscript{pro}) mediates the proteolytic processing of replicase polyproteins 1a and 1ab into functional proteins, playing an important role in viral replication. In this study, we demonstrated the expression of the SARS-CoV 3CL\textsuperscript{pro} in Escherichia coli and Vero cells, and then characterized the in vitro trans-cleavage and the cell-based cis-cleavage by the 3CL\textsuperscript{pro}. Mutational analysis of the 3CL\textsuperscript{pro} demonstrated the importance of His41, Cys145, and Glu166 in the substrate-binding site S1 for keeping the proteolytic activity. In addition, alanine substitution of the cleavage substrates indicated that Glu\textsubscript{166} in the substrates mainly determined the cleavage efficiency. Therefore, this study not only established the quantifiable and reliable assay for the in vitro and cell-based measurement of the 3CL\textsuperscript{pro} activity, but also characterized the molecular interaction of the SARS-CoV 3CL\textsuperscript{pro} with the substrates. The results will be useful for the rational development of the anti-SARS drugs.

\textcopyright 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: SARS-coronavirus; 3C-like protease; trans- and cis-cleavage; Substrate specificity

1. Introduction

Severe acute respiratory syndrome (SARS) with high fever, malaise, headache, dry cough, and a progress of generalized, interstitial infiltrates in the lung has recently been reported over 32 countries around the world, including Taiwan, China, Hong Kong, Vietnam, and Canada [1–4]. SARS was rapidly transmitted through aerosols, causing 8447 reported cases with 811 deaths worldwide in a short period from February to June, 2003 [5–7]. For successful control of the SARS outbreak, developing effective therapies and vaccines becomes medically important efforts.

A novel coronavirus (CoV), SARS-coronavirus (SARS-CoV), was identified as the etiological agent of the disease [1–4]. SARS-CoV particles contain a single positive-stranded RNA genome that is approximately 30 kb in length and has a 5’ cap structure and 3’ poly(A) tract [8–10]. The SARS-CoV genome encodes for replicase, spike, envelope, membrane, and nucleocapsid. The replicase gene encodes two large overlapping polyproteins (replicase 1a and 1ab, \(\sim 450\) and \(\sim 750\) kDa, respectively), including 3C-like protease (3CL\textsuperscript{pro}), RNA-dependent RNA polymerase, and RNA helicase for viral replication and transcription [11]. The SARS-CoV 3CL\textsuperscript{pro} mediates the proteolytic processing of replicase polyproteins 1a and 1ab into functional proteins, playing an important role in viral replication. Eleven cleavage sites of the 3CL\textsuperscript{pro} on the viral polyprotein have been mapped using the computer prediction based on the substrate conservation among CoV main proteases [12], being confirmed by the in vitro trans-cleavage of 11 substrate peptides [13]. Therefore, the SARS-CoV 3CL\textsuperscript{pro} becomes an attractive target for developing effective drugs against SARS.

In this study, we characterized the in vitro trans-cleavage and the cell-based cis-cleavage with the SARS-CoV 3CL\textsuperscript{pro} (Fig. 1A and B). For the trans-cleavage assay, the functional 3CL\textsuperscript{pro} and three mutants at the substrate-binding sites were used to test their proteolytic activity with the cleavage substrate-I (S-I), TVRLQAGNAT) fused at the N-terminus of the SARS-CoV non-structure protein 7 (nsp7). For the cis-cleavage assay, the in-frame construction of the 3CL\textsuperscript{pro}, the substrate-II (S-II), SAVLQSGFRK, and the luciferase (Luc) was transfected into the Vero cells. In addition, the mutations at the substrate conserved residues Leu and Glu were performed for the examination of the substrate specificity. In this study, the in vitro trans-cleavage and cell-based cis-cleavage activities of the SARS-CoV 3CL\textsuperscript{pro} had been determined using the quantitative methods of an Enzyme-Linked Immunosorbent Assay (ELISA) and a Luc assay, which will be useful for large-scale screening of inhibitors against SARS.

Abstract Severe acute respiratory syndrome (SARS) has been globally reported. A novel coronavirus (CoV), SARS-CoV, was identified as the etiological agent of the disease. SARS-CoV 3C-like protease (3CL\textsuperscript{pro}) mediates the proteolytic processing of replicase polyproteins 1a and 1ab into functional proteins, playing an important role in viral replication. In this study, we demonstrated the expression of the SARS-CoV 3CL\textsuperscript{pro} in Escherichia coli and Vero cells, and then characterized the in vitro trans-cleavage and the cell-based cis-cleavage by the 3CL\textsuperscript{pro}. Mutational analysis of the 3CL\textsuperscript{pro} demonstrated the importance of His41, Cys145, and Glu166 in the substrate-binding site S1 for keeping the proteolytic activity. In addition, alanine substitution of the cleavage substrates indicated that Glu\textsubscript{166} in the substrates mainly determined the cleavage efficiency. Therefore, this study not only established the quantifiable and reliable assay for the in vitro and cell-based measurement of the 3CL\textsuperscript{pro} activity, but also characterized the molecular interaction of the SARS-CoV 3CL\textsuperscript{pro} with the substrates. The results will be useful for the rational development of the anti-SARS drugs.

\textcopyright 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: SARS-coronavirus; 3C-like protease; trans- and cis-cleavage; Substrate specificity

1. Introduction

Severe acute respiratory syndrome (SARS) with high fever, malaise, headache, dry cough, and a progress of generalized, interstitial infiltrates in the lung has recently been reported over 32 countries around the world, including Taiwan, China, Hong Kong, Vietnam, and Canada [1–4]. SARS was rapidly
2. Materials and methods

2.1. Construction, expression, and purification of SARS-CoV 3CL<sup>pro</sup>

The 3CL<sup>pro</sup> gene located within the nucleotides 9985–10902 of the SARS-CoV TW1 strain genome (GenBank Accession No. AY291451) [14] was amplified using the reverse-transcriptase polymerase chain reaction (RT-PCR) with specific primers 5'-CCCCGATACCATGTGTTTTAGGAAGATGCGAGCTC-3' and 5'-GGTGCCTGAGTGGGAAGCCGCATATGATG-3' for the mutation of Glu166 and His41, Cys145, and Glu166 within the catalytic sites of the SARS-CoV 3CL<sup>pro</sup> protein were mutated by the PCR method similar to our previous report [16]. Site-directed mutagenesis was conducted by using paired complementary oligonucleotides for the desired point mutations. The samples from fractions of each purification step were then dissolved in 2× SDS-PAGE sample buffer without 2-mercaptoethanol and boiled for 10 min. Proteins were resolved on 12% SDS–PAGE gels and stained with Coomassie Brilliant Blue (Sigma). Moreover, the non-cleavage product of the fusion protein S-Insp7 by the 3CL<sup>pro</sup> and the mutants (H41A, C145A, and E166R) was captured onto the anti-HSV mAb coated microwells. The non-cleavage fusion protein was detected using the S protein-HRP conjugate and ABTS/ H<sub>2</sub>O<sub>2</sub> substrates. For cis-cleavage assay, the in-frame construction of the 3CL<sup>pro</sup>, the substrate-II, and Luc plus the pEGFP-N1 was transfected into the Vero cells. The Luc activity was referred to monitor the cis-cleavage of the fusion protein, the 3CL<sup>pro</sup>-S-H-Luc.

2.2. SDS–PAGE and Western blotting

The samples from fractions of each purification step were then dissolved in 2× SDS-PAGE sample buffer without 2-mercaptoethanol and boiled for 10 min. Proteins were resolved on 12% SDS–PAGE gels and stained with Coomassie Brilliant Blue (Sigma). Moreover, the electrophoretically separated proteins were transferred to nitrocellulose paper. The resultant blots were blocked with 5% skimmed milk and then reacted with the appropriately diluted Anti-His Tag monoclonal antibody (mAb) (Sero tec) for a 3-h incubation. The blots were then washed with 1% TBS containing 0.05% Tween 20 (TBST) three times and overlaid with a 1/5000 dilution of goat anti-mouse IgG antibodies conjugated with alkaline phosphatase (Perkin–Elmer Life Sciences, Inc.). Following a 1-h incubation at room temperature, the blots were developed with TNBT/BCIP (Gibco).

2.3. Azocasein digestion of SARS-CoV 3CL<sup>pro</sup>

The protease activity of the SARS-CoV 3CL<sup>pro</sup> was determined spectrophotometrically following the digestion of azocasein (Sigma) as the substrate [15]. 150 μl of samples was added to 150 μl of chromogen reagent containing 2% azocasein in 50 mM Tris–HCl, pH 8.5. After 2-h incubation at 37 °C, non-digested azocasein was precipitated by adding 350 μl of 10% trichloroacetic acid (TCA) (Merck). For determining the proteolytic activity, 350 μl of the resulting supernatants centrifuged at 10000 × g for 10 min, was mixed with 300 μl of 1 N NaOH, and then the absorbance of the above mixture at 440 nm was measured. The blank was obtained by precipitating the substrate plus the sample in TCA without incubation.

2.4. Site-directed mutagenesis of SARS-CoV 3CL<sup>pro</sup>

His41, Cys145, and Glu166 within the catalytic sites of the SARS-CoV 3CL<sup>pro</sup> protein were mutated by the PCR method similar to our previous report [16]. Site-directed mutagenesis was conducted by using paired complementary oligonucleotides for the desired point mutations to generate specific mutants within the SARS-CoV 3CL<sup>pro</sup> protein. The samples from fractions of each purification step were then dissolved in 2× SDS-PAGE sample buffer without 2-mercaptoethanol and boiled for 10 min. Proteins were resolved on 12% SDS–PAGE gels and stained with Coomassie Brilliant Blue (Sigma). Moreover, the electrophoretically separated proteins were transferred to nitrocellulose paper. The resultant blots were blocked with 5% skimmed milk and then reacted with the appropriately diluted Anti-His Tag monoclonal antibody (mAb) (Sero tec) for a 3-h incubation. The blots were then washed with 1% TBS containing 0.05% Tween 20 (TBST) three times and overlaid with a 1/5000 dilution of goat anti-mouse IgG antibodies conjugated with alkaline phosphatase (Perkin–Elmer Life Sciences, Inc.). Following a 1-h incubation at room temperature, the blots were developed with TNBT/BCIP (Gibco).
3CLpro protein constructs was confirmed using a DNA sequence analysis.

2.5. Construction and expression of SARS-CoV nsps fusion protein

The nsps7 gene located in the nucleotides 12937–13356 of the TW1 strain genome. The cleavage S-I (TVRLQAGNATE) for the 3CLpro protein located within the junction of ns6 and nsps7, being fused at the N-terminus of the nsps7 protein (Fig. 1A). The S-NSps7 gene was amplified using PCR with specific primers 5'-CCGGTTTCTGACTCAGTTGCTAACCTCGGACGGTCGT-3' and 5'-CGAAGCTTGGCGAGGAGTTACACGGCGATCTTTCCGCCCTT-3'. The forward primer mentioned above contained a BamHI restriction site and the reverse primers included a HindIII restriction site. Each RT-PCR product was digested with BamHI and HindIII, and then ligated into the BamHI/HindIII cleavage sites of the pET43.1B vector (Novagen). The resultant plasmids were transformed into the E. coli strain BL21(DE3). The S-NSps7 fusion protein expressed in E. coli was purified using the HisTrap Kit (Amersham).

2.6. In vitro trans-cleavage activity of the 3CLpro determined by ELISA

For determining the trans-acting proteolytic activity, the SARS-CoV 3CLpro reacted with the S-NSps7 fusion protein captured onto the microtiter plates (Fig. 1A). The wells of a 96-well plate were coated with 100 μl of diluted anti-HSV mAb (Novagen) and incubated overnight at 4°C. Following each incubation and subsequent layer of the ELISA, the wells were washed three times with TBST. After blocking by incubation with 5% skimmed milk in TBST for 2 h at room temperature (200 μl/well), 100 μl of the mixture containing the S-NSps7 fusion protein (10 μg/ml) and the 3CLpro (300 μg/ml) was added into anti-HSV mAb-coated wells for the 3-h incubation. The intact form of the S-NSps7 fusion protein was detected using the S protein conjugated to peroxidase (Novagen) for 1 h at room temperature. The ELISA products were developed with a chromogen solution containing 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and hydrogen peroxide. The relative trans-cleavage activity was calculated as 1 - (A4053CLpro)/A405no 3CLpro.

2.7. The cell-based cis-cleavage activity of the 3CLpro

For examining the cis-acting proteolytic assay, the 3CLpro was fused in-frame with a cleavage site and a Luc at the C-terminus (Fig. 1B). The 3CLpro gene was amplified using PCR with the paired primers 5'-ACGGCATCAGTTGCTAACCTCGGACGGTCGTAC-ACTCAGTTGCTAACCTCGGACGGTCGT-3' and 5'-GGTGGAATTCTGAGTTAGGAAAATGGCATTC-3'. The forward primer mentioned above contained a BamHI restriction site and the reverse primers included a HindIII restriction site. The RT-PCR product was digested with BamHI and HindIII, and then ligated into the BamHI/HindIII cleavage sites of the pET43.1B vector (Novagen). The purified SARS-CoV 3CLpro existed at more than 200 μg/ml concentration as a mixture of the inactive monomer (major) and the active dimer (minor) [13].

3.2. Azocasein hydrolysis of the SARS-CoV 3CLpro

To test the protease activity of the recombinant 3CLpro protein, the azocasein hydrolys activity in the transfected cells was measured using the dual Luciferase Reporter Assay System (Promega) and the Lumimeter TROPIX TR-717 (Applied Biosystems).

3.3. In vitro trans-cleavage activity of the 3CLpro

The proteolytic specificity of the SARS-CoV 3CLpro was examined using the in vitro trans-cleavage of the S-I (TVRLQAGNATE mapped at the junction of ns6 and nsps7). The S-I was fused in-frame with a Nus-Tag and an S-Tag at the N-terminus and the nsps7 and an HSV-Tag at the C-terminus (Fig. 1A). According to the structure knowledge [17], mutations of His-41 by Ala (H41A), Cys-145 by Ala (C145A), and Glu166 by Arg (E166R) within the substrate-binding site.
SI were also performed and tested the effects on the enzyme activity. The trans-cleavage of the S-I/nsp7 fusion protein (Nus Tag/S-Tag/S-I/nsp7/HSV-Tag) by the 3CLpro and the mutants was analyzed using the Western blotting with the S-protein conjugated to peroxidase. An immuno-band for the cleavage product, the Nus Tag/S-Tag protein, was detected in the trans-cleavage by 3CLpro, but not in the reactions by the 3CLpro mutants H41A, C145A, and E166R (data not shown). For quantification of the in vitro trans-cleavage, the mixture of the 3CLpro and the S-I/nsp7 fusion protein was incubated in the anti-HSV mAb-coated wells. Subsequently, the non-cleavage form of the S-I/nsp7 fusion protein was captured and then precipitated with the substrate plus the sample in TCA without incubation.

Fig. 2. SDS–PAGE (A), Western blotting (B), and enzyme activity (C) of the recombinant SARS-CoV 3CLpro in each purified step. (A) The supernatant of the sonicated cells transformed with the pET24a-3CLpro plasmid was purified by IMAC. 20 µl of each eluted fraction was analyzed by 12% SDS–PAGE with Coomassie blue staining. Lanes 2–9 indicated that the samples from the fractions eluted with 20, 40, 60, 100, 200, 300, 400, and 500 mM imidazole, respectively. Lane 1 was the molecular marker. kDa, kilodaltons. (B) 5 µl of each eluted fraction was analyzed by 12% SDS–PAGE, then electrophoretically transferred onto nitrocellulose paper. The blot was probed with mouse anti-His tag antibodies, and developed with an alkaline phosphatase-conjugated secondary antibody and NBT/BCIP substrates. Lanes 2–7 indicated that the samples from the fractions eluted with 60, 100, 200, 300, 400, and 500 mM imidazole, respectively. (C) The digestion of azocasein by the 3CLpro protein was measured at 440 nm. The blank was obtained by precipitating the substrate plus the sample in TCA without incubation.
detected using the ELISA with the S-protein conjugated to peroxidase (Fig. 3). The relative trans-cleavage ability revealed that the 3CLpro mutants H41A, C145A, and E166R lose more than 50% activity compared to the wild type 3CLpro. Furthermore, the enzyme activity of the 3CLpro was significantly inhibited by the serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (ABF) (Fig. 3). These results demonstrated the trans-cleavage specificity of the 3CLpro and the importantly functional role of the residues His41, Cys145, and Glu166 within the substrate-binding site S1.

3.4. cis-cleavage activity of the 3CLpro in the cell-based assays

For the cell-based cis-cleavage assay, the in-frame construction (Pro/S-II/Luc) of the 3CLpro, the S-II (SAVLQSGFRK), and the Luc plus pEGFP-N1 was transfected into Vero cells (Fig. 4D). Since the fusion of the firefly Luc with a more than 30 kDa protein fused as the N-terminus inhibited the serine protease inhibitor 4-((2-aminoethyl)benzenesulfonfyl fluoride hydrochloride (ABF) (Fig. 3). These results demonstrated the trans-cleavage specificity of the 3CLpro and the importantly functional role of the residues His41, Cys145, and Glu166 within the substrate-binding site S1.

4. Discussion

In this study, we demonstrated the expression and functional activity of the SARS-CoV 3CLpro in E. coli and Vero cells, and also characterized the trans- and cis-cleavage of the substrates TVRLQAGNAT and SAVLQSGFRK in the fusion proteins by the 3CLpro. In addition, we examined the active site SI and substrate specificity of the 3CLpro using site-directed mutagenesis, providing the insight into molecular recognition of the SARS-CoV 3CLpro with the substrates for the rational design of anti-SARS drugs.

Our results indicated that the recombinant SARS-CoV 3CLpro exists as a mixture of monomers (major) and dimers (minor) in the solutions (Fig. 2B), being in agreement with other studies on the recombinant protease of human CoV (HCoV) and the related porcine transmissible gastroenteritis (corona)virus (TGEV) [19,20]. Furthermore, alanine substitution at the Cys145–His41 catalytic dyad resulted in a significant loss of the 3CLpro enzyme activity (Fig. 3), revealing the importance of the Cys145–His41 catalytic dyad. According to the crystallographic data [17], the Glu166 in the substrate-binding subsite, S1, of the SARS-CoV 3CLpro has a salt bridge with His172 and hydrogen bonds with the NH group of the other monomer Ser1, being important for the substrate binding and the 3CLpro dimerization. Mutational analysis of Glu166 confirmed the importance of Glu166 in the enzymatic function of the SARS-CoV 3CLpro (Fig. 3). These results showed the important role of the substrate-binding subsite S1 in the anti-SARS drug design.

The identified cleavage site of the SARS-CoV 3CLpro contains a LQA(S,N) motif recognized by most other CoV proteases [12,21], which leads us to suggest that the proteolytic processing of the SARS-CoV replica polyproteins could be similar to those of other CoVs, such as HCoV and TGEV. Based on the conserved LQA(S,N) motif, Leu-P2 and Gln-P1 were selected for mutational analysis of the substrate specificity by the 3CLpro. Alanine substitution at Leu-P2 and Gln-P1 revealed that Gln-P1 dominatedly determined the cleavage efficiency of the substrates by the SARS-CoV 3CLpro (Fig. 4G). We will further characterize the substrate specificity by the systematically mutational analysis for the molecular-based design on the anti-SARS drugs.

In this study, we establish the in vitro trans-cleavage assay and the cell-based cis-cleavage assay with the recombinant 3CLpro protein. The azocasein and the substrate fusion protein Nus-Tag/S-Tag/S-I/nsp7/HSV-Tag and the 3CLpro was added into the anti-HSV mAb-coated wells. The inhibitor ABF was also added into the trans-cleavage assay. The non-cleavage S-I/nsp7 fusion protein captured onto 96-well plates with anti-HSV mAb was detected using the S protein-HRP conjugate and ABTS/H2O2 substrates. The ELISA product was measured at 405 nm. The relative trans-cleavage activity was calculated as 1-(A405nm/3CLpro)/(A405nm/3CLpro ABF).

Fig. 3. ELISA for the trans-cleavage of the S-I/nsp7 fusion protein by the 3CLpro and the mutants H41A, C145A, and E166R. After a 3-h incubation, the mixture of the fusion protein Nus-Tag/S-Tag/S-I/nsp7/HSV-Tag and the 3CLpro was added into the anti-HSV mAb-coated wells. The inhibitor ABF was also added into the trans-cleavage assay. The non-cleavage S-I/nsp7 fusion protein captured onto 96-well plates with anti-HSV mAb was detected using the S protein-HRP conjugate and ABTS/H2O2 substrates. The ELISA product was measured at 405 nm. The relative trans-cleavage activity was calculated as 1-(A405nm/3CLpro)/(A405nm/3CLpro ABF).

The identified cleavage site of the SARS-CoV 3CLpro contains a LQA(S,N) motif recognized by most other CoV proteases [12,21], which leads us to suggest that the proteolytic processing of the SARS-CoV replica polyproteins could be similar to those of other CoVs, such as HCoV and TGEV. Based on the conserved LQA(S,N) motif, Leu-P2 and Gln-P1 were selected for mutational analysis of the substrate specificity by the 3CLpro. Alamine substitution at Leu-P2 and Gln-P1 revealed that Gln-P1 dominantly determined the cleavage efficiency of the substrates by the SARS-CoV 3CLpro (Fig. 4G). We will further characterize the substrate specificity by the systematically mutational analysis for the molecular-based design on the anti-SARS drugs.

In this study, we establish the in vitro trans-cleavage assay and the cell-based cis-cleavage assay with the recombinant 3CLpro protein. The azocasein and the substrate fusion protein Nus-Tag/S-Tag/S-I/nsp7/HSV-Tag and the 3CLpro was added into the anti-HSV mAb-coated wells. The inhibitor ABF was also added into the trans-cleavage assay. The non-cleavage S-I/nsp7 fusion protein captured onto 96-well plates with anti-HSV mAb was detected using the S protein-HRP conjugate and ABTS/H2O2 substrates. The ELISA product was measured at 405 nm. The relative trans-cleavage activity was calculated as 1-(A405nm/3CLpro)/(A405nm/3CLpro ABF).

4. Discussion

In this study, we demonstrated the expression and functional activity of the SARS-CoV 3CLpro in E. coli and Vero cells, and also characterized the trans- and cis-cleavage of the substrates TVRLQAGNAT and SAVLQSGFRK in the fusion proteins by the 3CLpro. In addition, we examined the active site SI and substrate specificity of the 3CLpro using site-directed mutagenesis, providing the insight into molecular recognition of the SARS-CoV 3CLpro with the substrates for the rational design of anti-SARS drugs.

Our results indicated that the recombinant SARS-CoV 3CLpro exists as a mixture of monomers (major) and dimers (minor) in the solutions (Fig. 2B), being in agreement with other studies on the recombinant protease of human CoV (HCoV) and the related porcine transmissible gastroenteritis (corona)virus (TGEV) [19,20]. Furthermore, alanine substitution at the Cys145–His41 catalytic dyad resulted in a significant loss of the 3CLpro enzyme activity (Fig. 3), revealing the importance of the Cys145–His41 catalytic dyad. According to the crystallographic data [17], the Glu166 in the substrate-binding subsite, S1, of the SARS-CoV 3CLpro has a salt bridge with His172 and hydrogen bonds with the NH group of the other monomer Ser1, being important for the substrate binding and the 3CLpro dimerization. Mutational analysis of Glu166 confirmed the importance of Glu166 in the enzymatic function of the SARS-CoV 3CLpro (Fig. 3). These results showed the important role of the substrate-binding subsite S1 in the anti-SARS drug design.

The identified cleavage site of the SARS-CoV 3CLpro contains a LQA(S,N) motif recognized by most other CoV proteases [12,21], which leads us to suggest that the proteolytic processing of the SARS-CoV replica polyproteins could be similar to those of other CoVs, such as HCoV and TGEV. Based on the conserved LQA(S,N) motif, Leu-P2 and Gln-P1 were selected for mutational analysis of the substrate specificity by the 3CLpro. Alamine substitution at Leu-P2 and Gln-P1 revealed that Gln-P1 dominantly determined the cleavage efficiency of the substrates by the SARS-CoV 3CLpro (Fig. 4G). We will further characterize the substrate specificity by the systematically mutational analysis for the molecular-based design on the anti-SARS drugs.

In this study, we establish the in vitro trans-cleavage assay and the cell-based cis-cleavage assay with the recombinant 3CLpro protein. The azocasein and the substrate fusion protein Nus-Tag/S-Tag/S-I/nsp7/HSV-Tag and the 3CLpro was added into the anti-HSV mAb-coated wells. The inhibitor ABF was also added into the trans-cleavage assay. The non-cleavage S-I/nsp7 fusion protein captured onto 96-well plates with anti-HSV mAb was detected using the S protein-HRP conjugate and ABTS/H2O2 substrates. The ELISA product was measured at 405 nm. The relative trans-cleavage activity was calculated as 1-(A405nm/3CLpro)/(A405nm/3CLpro ABF).
the molecular interaction of the SARS-CoV 3CLpro with the substrates, but also provides reliable assays for screening the anti-SARS drugs.

Acknowledgements: We thank the National Science Council (Taiwan) and China Medical University for financial supports (NSC 92-2314-B-039-030, NSC 92-2751-B-039-009-Y, and CMU92-MT-03).

References


