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-Hsuh 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 Virolology 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

Available 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 cell-based cis-cleavage by the 3CL\textsuperscript{pro}. Mutational analysis of the 3CL\textsuperscript{pro} demonstrated the importance of His41, Cys145, and Gln166 in the substrate-binding site S1 for keeping the proteolytic activity. In addition, alanine substitution of the cleavage substrates indicated that Gln166 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.

© 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, ~450 and ~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 replicate 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 Gln 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.

* Corresponding authors. Fax: +886-4-22057414 (C.-W. Lin). E-mail address: cwlil@mail.cmu.edu.tw (C.-W. Lin).

Abbreviations: SARS, severe acute respiratory syndrome; CoV, coronavirus; 3CL\textsuperscript{pro}, 3C-like protease; S-I (II), substrate-I (II); Luc, luciferase

* Corresponding authors. Fax: +886-4-22057414 (C.-W. Lin). E-mail address: cwlil@mail.cmu.edu.tw (C.-W. Lin).

Abbreviations: SARS, severe acute respiratory syndrome; CoV, coronavirus; 3CL\textsuperscript{pro}, 3C-like protease; S-I (II), substrate-I (II); Luc, luciferase

0014-5793/$22.00 © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2004.08.017
2. Materials and methods

2.1. Construction, expression, and purification of SARS-CoV 3CL\textsuperscript{pr}

The 3CL\textsuperscript{pr} 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'-CCCGGATCCAGTG-GTTTTAGGAAAATGGCATTC-3' and 5'-GGTGCTCGAGTTGG-AAGGTAACACCAGAGCATTG-3'. The forward primer mentioned above contained an EcoRI restriction site and the reverse primers included an XhoI restriction site. Each RT-PCR product was digested with EcoRI and XhoI, and then ligated into the EcoRI/XhoI cleavage sites of the pET24a vector (Novagen) and expressed as a histidine tag fusion protein. The fusion protein was then expressed by the addition of 4 mM isopropyl-\beta-D-thiogalactopyranoside (IPTG) in the presence of 100 \( \mu \)g/ml of kanamycin per ml at 37 \(^\circ\)C. Finally, the bacteria were harvested by centrifugation for 20 min. The concentration of the purified protein was determined using the Bio-Rad protein assay reagent.

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) (Serotec) 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\textsuperscript{pr}

The protease activity of the SARS-CoV 3CL\textsuperscript{pr} was determined spectrophotometrically following the digestion of azocasein (Sigma) as the substrate [15]. 150 \( \mu \)l of samples was added to 150 \( \mu \)l of chromogen reagent containing 2% azocasein in 50 mM Tris–HCl, pH 8.5. After 2-h incubation at 37 \(^\circ\)C, non-digested azocasein was precipitated by adding 350 \( \mu \)l of 10% trichloroacetic acid (TCA) (Merck). For determining the proteolytic activity, 350 \( \mu \)l of the resulting supernatants centrifuged at 10000 \( \times \) g for 10 min, was mixed with 300 \( \mu \)l 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\textsuperscript{pr}

His41, Cys145, and Glu166 within the catalytic sites of the SARS-CoV 3CL\textsuperscript{pr} 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 mutations into the SARS-CoV 3CL\textsuperscript{pr} protein. The pairs of primers used were 5'-TGTTGGAAGCCGCATATGATG-3' and 5'-AATGGATCAGCCGGTAGTGTT-3' for the substitution of His41 with alanine, 5'-AATTGGATCAGCCGGTAGTGTT-3' and 5'-GGTGCTCGAGTTGG-AAGGTAACACCAGAGCATTG-3' for the replacement of Cys145 with alanine, and 5'-AACTACTACCGGCTGATCCATT-3' and 5'-GCAAATGACGGCTCTTGGACA-3' for the mutation of Glu166 with arginine. The correct sequence of each mutant of the SARS-CoV...
3CL\textsuperscript{pro} protein constructs was confirmed using a DNA sequence analysis.

2.5. Construction and expression of SARS-CoV nsP7 fusion protein

The nsP7 gene located in the nucleotides 12 937–13 356 of the TW1 strain genome. The cleavage S-I (TVRQLAGNATE) for the 3CL\textsuperscript{pro} protein located within the junction of nsP6 and nsP7, being fused at the N-terminus of the nsP7 protein (Fig. 1A). The S-nsP7 gene was amplified using PCR with specific paired primers 5'-CGTGGATCCGGCTACAGTTTCCATCGGCT-3' and 5'-CGacaagctGCAGGATTTTGTCATCACAAGTCA-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-nsP7 fusion protein expressed in E. coli was purified using the HisTrap Kit (Amersham).

2.6. In vitro trans-cleavage activity of the 3CL\textsuperscript{pro} determined by ELISA

For determining the trans-acting proteolytic assay, the SARS-CoV 3CL\textsuperscript{pro} reacted with the S-nsP7 fusion protein captured onto the microtiter plate (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-nsP7 fusion protein (10 μg/ml) and the 3CL\textsuperscript{pro} (300 μg/ml) was added into anti-HSV mAb-coated wells for the 3-h incubation. The intact S-I was fused in-frame with a Nus-Tag and an HSV-Tag at the C-terminus (TVRLQAGNATE mapped at the junction of nsp6 and nsp7). According to the structure knowledge [17], the N-terminus and the nsp7 and an HSV-Tag at the C-terminus of the S-I were designed as Pro/S-II(Q/L) and Pro/S-II(Q/L), respectively.

2.7. The cell-based cis-cleavage activity of the 3CL\textsuperscript{pro}

For examining the cis-acting proteolytic assay, the 3CL\textsuperscript{pro} was fused in-frame with a cleavage site and a Luciferase reporter assay system (Promega) and the LuminoMeter TROPiX TR-717 (Applied Biosystems).

3. Results

3.1. Expression and purification of the recombinant SARS-CoV 3CL\textsuperscript{pro} in E. coli

To examine the expression of the 3CL\textsuperscript{pro} protein in E. coli, the C-terminal His tagged 3CL\textsuperscript{pro} protein was detected using Western blotting with anti-His Tag monoclonal antibody. Western blot revealed that a 34-kDa protein in the supernatant and the pellet fractions was found in the recombinant E. coli (data not shown). The 34-kDa recombinant protein was in agreement with the theoretical molecular weight (33.8 kDa) of the recombinant 3CL\textsuperscript{pro} fusion protein using the Compute pl/Mw tool (http://www.expasy.org).

Subsequently, the soluble 3CL\textsuperscript{pro} protein expressed in the E. coli was harvested from the supernatant of the sonicated cells and then purified using the immobilized-metal affinity chromatography (IMAC). The Coomassie blue-stained gel revealed that one major band of the recombinant 3CL\textsuperscript{pro} protein was eluted with imidazole ranging from 100 to 500 mM (Fig. 2A, lanes 5–9). The high purity of the 3CL\textsuperscript{pro} was observed in the last eluted fraction with 500 mM imidazole (Fig. 2A, lane 9). Interestingly, Western blotting demonstrated that an about 68-kDa immuno-reactive band, except a 34-kDa band, was found at more than 200 μg/ml concentration of our purified 3CL\textsuperscript{pro}, which was in the eluted fraction with imidazole ranging from 300 to 500 mM (Fig. 2B, lanes 5–7). However, no 68-kDa immuno-reactive band was observed at lower than 200 μg/ml concentration, being in the eluted fraction with 200 mM imidazole (Fig. 2B, lane 4). The result was in agreement with a previous report in which the purified SARS-CoV 3CL\textsuperscript{pro} existed at more than 200 μg/ml protein concentration as a mixture of the inactive monomer (major) and the active dimer (minor) [13].

3.2. Azocasein hydrolysis of the SARS-CoV 3CL\textsuperscript{pro}

To test the protease activity of the recombinant 3CL\textsuperscript{pro} protein, the azocasein hydrolysis of the 3CL\textsuperscript{pro} protein in each eluted fraction was further performed (Fig. 2C). The azocasein proteolytic profile revealed that the 3CL\textsuperscript{pro} protein with a high purity eluted at 500 mM imidazole has highest proteolytic activity compared to those eluted fractions at 20, 40, 60, 100, and 300 mM imidazole (Fig. 2C). Moreover, the azocasein proteolytic assay showed a dose-dependent ability of the 3CL\textsuperscript{pro} protein using the serial 2-fold dilution ranging from 200 to 800 μg/ml (data not shown). The results showed the enzyme activity of the purified 3CL\textsuperscript{pro} protein.

3.3. In vitro trans-cleavage activity of the 3CL\textsuperscript{pro}

The proteolytic specificity of the SARS-CoV 3CL\textsuperscript{pro} was examined using the in vitro trans-cleavage of the S-I (TVRQLAGNATE mapped at the junction of nsP6 and nsP7). The S-I was fused in-frame with a Nus-Tag and an HSV-Tag at the N-terminus and the nsP7 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

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.
cis-cleavage with a more than 30 kDa protein fused as the N-terminus into Vero cells (Fig. 4D). Since the fusion of the firefly Luc (LQSGFRK), and the Luc plus pEGFP-N1 was transfected with anti-HSV mAb demonstrated that three immuno-

conserved residues Leu-P2 and Gln-P1, designed as Pro/S-II(L

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 peroxidase (Fig. 3). The relative trans-cleavage activity of the 3CL pro 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 3CL pro 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 3CL pro in the cell-based assays

For the cell-based cis-cleavage assay, the in-frame construction (Pro/S-II/Luc) of the 3CL pro, the S-II (SAVLQAGGAT and SAVLQGFRK in the fusion proteins with His172 and hydrogen bonds with the NH group of the other monomer Ser1, being important for the substrate binding and the 3CL pro dimerization. Mutational analysis of Glu166 confirmed the importance of Glu166 in the enzymatic function of the SARS-CoV 3CL pro (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 3CL pro 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 replicate 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 3CL pro. Alanine substitution at the Cys145–His41 catalytic dyad resulted in a significant loss of the 3CL pro 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 3CL pro 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 3CL pro dimerization. Mutational analysis of Glu166 confirmed the importance of Glu166 in the enzymatic function of the SARS-CoV 3CL pro (Fig. 3). These results showed the important role of the substrate-binding subsite S1 in the anti-SARS drug design.

In this study, we demonstrated the expression and functional activity of the SARS-CoV 3CL pro 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 3CL pro. In addition, we examined the active site SI and substrate specificity of the 3CL pro using site-directed mutational analysis indicated that the conserved residue Gln at the P1 position mainly determined the substrate cleavage efficiency of the 3CL pro.

4. Discussion

In this study, we demonstrated the expression and functional activity of the SARS-CoV 3CL pro 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 3CL pro. In addition, we examined the active site SI and substrate specificity of the 3CL pro using site-directed mutational analysis indicated that the conserved residue Gln at the P1 position mainly determined the substrate cleavage efficiency of the 3CL pro.

In this study, we established the in vitro trans-cleavage assay and the cell-based cis-cleavage assay with the recombinant 3CL pro protein. The azocasein and the substrate fusion protein Nus-Tag/S-Tag/S-I/nsp7/HSV-Tag were used as the substrates in the in vitro trans-cleavage assay, providing the rapid and quantifiable assay for large-scale screening of SARS-CoV 3CL pro inhibitors. Furthermore, the Luc activity referred to the cell-based cis-cleavage activity will be useful for the examination of the inhibitory effects on the SARS-CoV 3CL pro in the Vero cells. Therefore, this study not only characterizes the cells carrying Pro/S-II(L→A)/Luc (23 972 ± 582 light units) and Pro/S-II(Q→A)/Luc (37 850 ± 282 light units), respectively (Fig. 4G). The results demonstrated the cis-cleavage of the fusion protein 3CL pro/substrate-II/Luc. Moreover, mutational analysis indicated that the conserved residue Gln at the P1 position mainly determined the substrate cleavage efficiency of the 3CL pro.
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

[1] Poutanen, S.M., Low, D.E., Henry, B., Finkelstein, S., Rose, D., Green, K., Tellier, R., Draker, R., Adachi, D., Ayers, M., Chan, A.K., Skowronski, D.M., Salit, I., Simor, A.E., Slutsky, A.S., Doyle, P.W., Krajden, M., Petric, M., Brunham, R.C. and McCleary, A.J. (2003) N. Engl. J. Med. 348, 1995–2005.

[2] Lee, N., Hui, D., Wu, A., Chan, P., Cameron, P., Joynt, G.M., Ahuja, A., Yung, M.Y., Leung, C.B., To, K.F., Lui, S.F., Szeto, C.C., Chung, S. and Sung, J.J. (2003) N. Engl. J. Med. 348, 1986–1994.

[3] Tsang, K.W., Ho, P.L., Ooi, G.C., Yee, W.K., Wang, T., Chan-Yeung, M., Lam, W.K., Seto, W.H., Yam, L.Y., Cheung, T.M., Wong, P.C., Lam, B., Ip, M.S., Chan, J., Yuen, K.Y. and Lai, K.N. (2003) N. Engl. J. Med. 348, 1977–1985.

[4] Hsueh, P.R., Chen, P.J., Hsiao, C.H., Yeh, S.H., Cheng, W.C., Wang, J.L., Chiang, B.L., Chang, S.C., Chang, F.Y., Wong, W.W., Kao, C.L. and Yang, P.C. (2004) Emerg. Infect. Dis. 10, 489–493.

[5] Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., Rollin, P.E., Dowell, S.F., Ling, A.E., Humphrey, C.D., Shieh, W.J., Guarnier, J., Paddock, C.D., Rota, P., Fields, B., DeRisi, J., Yang, J.Y., Cox, N., Hughes, J.M., LeDuc, J.W., Bellini, W.J. and Anderson, L.J. (2003) N. Engl. J. Med. 348, 1953–1966.

[6] Peiris, J.S., Chu, C.M., Cheng, V.C., Chan, K.S., Hung, I.F., Poon, L.L., Law, K.I., Tang, S.Y., Hon, T.Y., Chan, C.S., Chan, K.H., Ng, J.S., Zheng, B.J., Ng, W.L., Lai, R.W., Guan, Y. and Yuen, K.Y. (2003) Lancet 361, 1767–1772.

[7] Drosten, C., Gunther, S., Preiser, W., van der Werf, S., Brodt, H.R., Becker, S., Rabenau, H., Panning, M., Klenk, H.D., Fouchier, R.A., Berger, A., Gunther, A., Cattarino, L., Krammer, F., Mühle, M., Schnitz, A.D., Schmitz, H. and Doerr, H.W. (2003) N. Engl. J. Med. 348, 1967–1976.

[8] Lai, M.M.C. and Holmes, K.V. (2001) in: Fields Virology (Knipe, D.M. and Howley, P.M., Eds.), Lippincott Williams and Wilkins, New York.
Enjuanes, L., Brian, D., Cavanagh, D., Holmes, K., Lai, M.M.C., Laude, H., Masters, P., Rottier, P., Siddell, S.G., Spaan, W.G.M., Taguchi, F. and Talbot, P. (2000) in: Virus Taxonomy (van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Mayo, M.A., McGeoch, D.J., Pringle, C.R. and Wickner, R.B., Eds.), Academic Press, New York.

Holmes, K.V. (2001) in: Fields Virology (Knipe, D.M. and Howley, P.M., Eds.), Lippincott Williams and Wilkins, New York.

Ziebuhr, J., Snijder, E.J. and Gorbaleyna, A.E. (2000) J. Gen. Virol. 81, 853–879.

Gao, F., Ou, H.Y., Chen, L.L., Zheng, W.X. and Zhang, C.T. (2003) FEBS Lett. 553, 451–456.

Fan, K., Wei, P., Feng, Q., Chen, S., Huang, C., Ma, L., Lai, B., Pei, J., Liu, Y., Chen, J. and Lai, L. (2004) J. Bio. Chem. 279, 1637–1642.

Hsueh, P.R., Hsiao. C.H., Yeh, S.H., Wang, W.K., Chen, P.J., Wang, J.T., Chang, S.C., Kao, C.L. and Yang, P.C. (2003) Emerg. Infect. Dis. 9, 1163–1167.

Tomarelli, R.M., Charney, J. and Harding, M.L. (1949) J. Lab. Clin. Med. 34, 428–433.

Lin, C.W. and Wu, S.C. (2003) J. Virol. 77, 2600–2606.

Yang, H., Yang, M., Ding, Y., Liu, Y., Lou, Z., Zhou, Z., Sun, L., Mo, L., Ye, S., Pang, H., Gao, G.F., Anand, K., Bartlam, M., Hilgenfeld, R. and Rao, Z. (2003) Proc. Natl. Acad. Sci. USA 100, 13190–13195.

Joubert, P., Pautigny, C., Madelaine, M.F. and Rasschaert, D. (2000) J. Gen. Virol. 81, 481–488.

Anand, K., Palm, G.J., Mesters, J.R., Siddell, S.G., Ziebuhr, J. and Hilgenfeld, R. (2002) EMBO J. 21, 3213–3224.

Anand, K., Ziebuhr, J., Wadhwnani, P., Mesters, J.R. and Hilgenfeld, R. (2003) Science 300, 1763–1767.

Hegyi, A. and Ziebuhr, J. (2002) J. Gen. Virol. 83, 595–599.