Proteomic analysis of up-regulated proteins in human promonocyte cells expressing severe acute respiratory syndrome coronavirus 3C-like protease

Chien-Chen Lai\textsuperscript{1,2}, Ming-Jia Jou\textsuperscript{3}, Shiuan-Yi Huang\textsuperscript{1}, Shih-Wein Li\textsuperscript{4}, Lei Wan\textsuperscript{1}, Fuu-Jen Tsai\textsuperscript{1*} and Cheng-Wen Lin\textsuperscript{4,5}

\textsuperscript{1} Department of Medical Genetics and Medical Research, China Medical University Hospital, Taichung, Taiwan
\textsuperscript{2} Institute of Molecular Biology, National Chung Hsing University, Taichung, Taiwan
\textsuperscript{3} Department of Anatomy, School of Medicine, China Medical University, Taichung, Taiwan
\textsuperscript{4} Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung, Taiwan
\textsuperscript{5} Clinical Virology Laboratory, Department of Laboratory Medicine, China Medical University Hospital, Taichung, Taiwan

The pathogenesis of severe acute respiratory syndrome coronavirus (SARS CoV) is an important issue for treatment and prevention of SARS. Previously, SARS CoV 3C-like protease (3CLpro) has been demonstrated to induce apoptosis via the activation of caspase-3 and caspase-9 (Lin, C. W., Lin, K. H., Hsieh, T. H., Shiu, S. Y. et al., FEMS Immunol. Med. Microbiol. 2006, 46, 375–380). In this study, proteome analysis of the human promonocyte HL-CZ cells expressing SARS CoV 3CLpro was performed using 2-DE and nanoscale capillary LC/ESI quadrupole-TOF MS. Functional classification of identified up-regulated proteins indicated that protein metabolism and modification, particularly in the ubiquitin proteasome pathway, was the main biological process occurring in SARS CoV 3CLpro-expressing cells. Thirty-six percent of identified up-regulated proteins were located in the mitochondria, including apoptosis-inducing factor, ATP synthase beta chain and cytochrome c oxidase. Interestingly, heat shock cognate 71-kDa protein (HSP70), which antagonizes apoptosis-inducing factor was shown to down-regulate and had a 5.29-fold decrease. In addition, confocal image analysis has shown release of mitochondrial apoptogenic apoptosis-inducing factor and cytochrome c into the cytosol. Our results revealed that SARS CoV 3CLpro could be considered to induce mitochondrial-mediated apoptosis. The study provides system-level insights into the interaction of SARS CoV 3CLpro with host cells, which will be helpful in elucidating the molecular basis of SARS CoV pathogenesis.

Keywords:
2-DE / 3C-like protease / MS / Severe acute respiratory syndrome (SARS) coronavirus

1 Introduction

A novel virus, severe acute respiratory syndrome (SARS)-associated coronavirus (SARS CoV) is rapidly transmitted through aerosols, causing 8447 reported SARS cases with...
811 deaths worldwide in a short period from February to June, 2003 [1–5]. The SARS patients had manifested symptoms, like bronchial epithelial denudation, loss of cilia, multinucleated syncytial cells and squamous metaplasia in their lung tissue [6, 7]. Other studies have shown that SARS CoV replicates in Vero-E6 cells with cytopathic effects [8, 9], and induces AKT signaling-mediated cell apoptosis [10].

SARS CoV particles contain an approximately 30-kbp positive-stranded RNA genome with a 5' cap structure and a 3' poly(A) tract [11–13]. The SARS CoV genome encodes replicase, spike, envelope, membrane, and nucleocapsid proteins. The replicase gene encodes two large overlapping polypeptides (replicase 1a and 1ab, ~450 and ~750 kDa, respectively), including 3C-like protease (3CLpro), RNA-dependent RNA polymerase, and RNA helicase for viral replication and transcription [14]. The SARS CoV 3CLpro mediates the proteolytic processing of replicase 1a and 1ab into functional proteins, playing an important role in viral replication. Therefore, the SARS CoV 3CLpro is an attractive target for developing effective drugs against SARS [12–14]. Recently, a SARS CoV 3CLpro-interacting cellular protein, vacuolar-H+ ATPase (V-ATPase) G1 subunit with a 3CLpro cleavage site-like motif was identified, affecting the intracellular pH in 3CLpro-expressing cells [15]. In human promonocyte cells, SARS CoV CoV 3CLpro has been demonstrated to induce apoptosis via caspase-3 and caspase-9 activities [16]. In addition, 3C protease of picornaviruses poliovirus, enterovirus 71 and rhinovirus have been demonstrated to be associated with host translation shutoff by cleaving the translation initiation factor eIF4GI and the poly(A)-binding protein (PABP) [17], and inactivation of NF-kappaB function associated with host translation shutoff by cleaving the poly(A)-binding protein (PABP) [17], and inactivation of NF-kappaB function.

In the post-genomic era, the combination of 2-DE and MS has provided an alternative approach to examine a comparative analysis of proteomic profiling during viral infection, allowing new insights into cellular mechanisms involved in viral pathogenesis [19–24]. The 2-DE/MS proteomic technologies have been used to analyze the protein profiles of plasma from SARS patients [19, 24], and to differentiate up-regulated and down-regulated proteins identified in the 3CLpro-expressing cells. Therefore, investigating pathogenesis of SARS CoV 3CLpro has become an important issue.

In the post-genomic era, the combination of 2-DE and MS has provided an alternative approach to examine a comparative analysis of proteomic profiling during viral infection, allowing new insights into cellular mechanisms involved in viral pathogenesis [19–24]. The 2-DE/MS proteomic technologies have been used to analyze the protein profiles of plasma from SARS patients [19, 24], and to differentiate up-regulated and down-regulated proteins identified in the SARS CoV-infected African green monkey kidney cells [20]. To identify proteomic alternations induced by SARS CoV 3CLpro, the combination of 2-DE and MS can be performed for quantitative analysis and identification of the unique protein profiling in the transfected cells-expressing 3CLpro.

In this study, we intended to investigate the comparative proteome analysis of human promonocyte HL-CZ cells in the presence and absence of SARS CoV 3CLpro. Seventy-three up-regulated and 21 down-regulated proteins identified in the 3CLpro-expressing cells were categorized according to their subcellular location, biological process and biological pathway based upon the PANTHER classification system (http://www.pantherdb.org/). Functional analysis of up-regulated proteins identified in the 3CLpro-expressing cells was further examined using immunoblot analysis and confocal microscopy.

2 Materials and methods

2.1 Cell culture

In our previous study [16], human promonocyte HL-CZ cell clones co-transfected with the plasmid pc3CLpro plus indicator vector pEGFP-N1 were established for 3CLpro-expressing cells, whereas human promonocyte HL-CZ cell clones co-transfected with the plasmid pcDNA3.1 plus indicator vector pEGFP-N1 were used as mock cells. The transfected cells were incubated with RPMI 1640 medium containing 10% FBS and 800 μg/mL of antibiotic G418. For determining expression of SARS CoV 3CLpro, the transfected cells were analyzed using Western blotting. The cell lysates were dissolved in 2X SDS-PAGE sample buffer without 2-mercaptoethanol, and boiled for 10 min. Proteins were resolved on 12% SDS-PAGE gels and transferred to NC paper. The resultant blots were blocked with 5% skim milk, and then reacted with appropriately diluted mouse mAb anti-His tag (Serotec), anti-Rpt4 (26S protease regulatory subunit 6A) (abcam) or rabbit anti-apoptosis-inducing factor (Sigma) for a 3-h incubation. The blots were then washed with TBST three times and overlaid with a 1/5000 dilution of alkaline phosphatase-conjugated with secondary antibodies. Following 1-h incubation at room temperature, blots were developed with TNBT/BCIP (Gibco).

2.2 2-DE and protein spot analysis

For 2-DE, mock cells and 3CLpro-expressing cells were harvested, washed twice with ice-cold PBS, and then extracted with lysis buffer containing 8 M urea, 4% CHAPS, 2% pH 3–10 non-linear (NL) IPG buffer (GE Healthcare), and the Complete, Mini, EDTA-free protease inhibitor mixture (Roche). After a 3-h incubation at 4°C, the cell lysates were centrifuged for 15 min at 16 000 g. The protein concentration of the resulting supernatants was measured using the BioRad Protein Assay (BioRad, Hercules, CA, USA). Protein sample (100 μg) was diluted with 350 μL of rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3–10 NL, 18 mM DTT, 0.002% bromophenol blue), and then applied to the nonlinear Immobiline DryStrips (17 cm, pH 3–10; GE Healthcare). After the run of 1-D IEF on a Multiphor II system (GE Healthcare), the gel strips were incubated for 30 min in the equilibration solution I (6 M urea, 2% SDS, 30% glycerol, 1% DTT, 0.002% bromophenol blue, 50 mM Tris-HCl, pH 8.8), and for another 30 min in the equilibration solution II (6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide, 0.002% bromophenol blue, 50 mM Tris-HCl, pH 8.8). Subsequently, the IPG gels were transferred to the top of 12% polyacrylamide gels (20 × 20 cm × 1.0 mm) for
the secondary dimensional run at 15 mA, 300 V for 14 h. Separated protein spots were fixed in the fixing solution (40% ethanol and 10% glacial acetic acid) for 30 min, stained on the gel with silver nitrate solution for 20 min, and then scanned by GS-800 imaging densitometer with PDQuest software version 7.1.1 (BioRad). Data from three independently stained gels of each sample were exported to Microsoft Excel for creation of the correction graphs, spot intensity graphs and statistical analysis.

2.3 In-gel digestion

The modified in-gel digestion method based on previous reports [25, 26] was performed for nanoelectrospray MS. Briefly, each spot of interest in the silver-stained gel was sliced and put into the microtube, and then washed twice with 50% ACN in 100 mM ammonium bicarbonate buffer (pH 8.0) for 10 min at room temperature. Subsequently, the excised-gel pieces were soaked in 100% ACN for 5 min, dried in a lyophilizer for 30 min and rehydrated in 50 mM ammonium bicarbonate buffer (pH 8.0) containing 10 μg/mL trypsin at 30°C for 16 h. After digestion, the peptides were extracted from the supernatant of the gel elution solution (50% ACN in 5.0% TFA), and dried in a vacuum centrifuge.

2.4 Nanoelectrospray MS and database search

The proteins were identified using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTARXL quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA, USA). The peptides were separated using an RP C18 capillary column (15 cm x 75 μm id) with a flow rate of 200 nL/min, and eluted with a linear ACN gradient from 10–50% ACN in 0.1% formic acid for 60 min. The eluted peptides from the capillary column were sprayed into the MS by a PicoTip electrospray tip (FS360-20-10-D-20; New Objective, Cambridge, MA, USA). Data acquisition from Q-TOF was performed using the automatic Information Dependent Acquisition (IDA; Applied Biosystem/MDS Sciex). Proteins were identified by the nanoLC-MS/MS spectra by searching against NCBI databases for exact matches using the ProID search program (http://www.matrixscience.com) [27]. A Homo sapiens taxonomy restriction was used and the mass tolerance of both precursor ion and fragment ions was set to ± 0.3 Da. Carbamidomethyl cysteine was set as a fixed modification, while serine, threonine, tyrosine phosphorylation and other modifications were set as variable modifications. The protein function and subcellular location were annotated using the Swiss-Prot (http://us.expasy.org/sprot/) database. The proteins were also categorized according to their biological process and pathway using the PANTHER classification system (http://www.pantherdb.org) as described in the previous studies [28–30].

2.5 Immunocytochemistry

For determining subcellular localization, HL-CZ cells were transiently co-transfected with p3CLpro or pcDNA3.1 plus a mitochondrial localization vector pDsRed-Mito (Clontech) using the GenePorter reagent. After a 3-day incubation, the cells were fixed on glass coverslips with ice-cold acetone for 4 min, and blocked with 1% BSA. The co-transfected cells were subsequently incubated with mouse mAb anti-His tag, anti-cytochrome c, or rabbit anti-apoptosis-inducing factor (Sigma) at 4°C overnight. After washing, the cells were incubated with FITC-conjugated goat anti-mouse immunoglobulin or anti-rabbit immunoglobulin at room temperature for 2 h. Confocal image analysis of the cells was performed using Leica TCS SP2 AOBS laser-scanning microscopy (Leica Microsystems, Heidelberg, Germany).

3 Results

3.1 Comparison of differential protein expression between mock cells and SARS CoV 3CLpro-expressing cells

To identify specific cell responses to SARS CoV 3CLpro, the differential expression of proteins in mock cells and 3CLpro-expressing cells were analyzed using 2-DE and nanoscale capillary LC/ESI Q-TOF MS. After confirming expression of SARS CoV 3CLpro in the transfected cells as previously described [16], protein extracts prepared from mock cells and 3CLpro-expressing cells were separated using 2-DE. The resolved protein spots in gels were presented using silver staining (Fig. 1). About 1000 protein spots in the p/l range of 3.2 to 10 and the molecular weight range of 14 to 97.4 kDa were detected on the gels of mock (Fig. 1A) and 3CLpro-expressing cells (Fig. 1B), respectively. For comparison, three independent 2-DE images of each protein extract from three independent cell cultures of mock cells and 3CLpro-expressing cells were selected for statistical analysis. Protein profiling revealed that 154 ± 15 up-regulated proteins and 141 ± 12 down-regulated proteins in SARS CoV 3CLpro-expressing cells were determined using GS-800 imaging densitometer with PDQuest software (Fig. 1). After the statistical analysis with Student’s t-test, 75 up-regulated proteins (Spot ID number between 1 and 75) showed a statistically significant 1.5-fold increase in spot intensity (p<0.05) (Table 1), whereas 21 down-regulated proteins (Spot ID number between 76 and 96) had a statistically significant 2.0-fold decrease in 3CLpro-expressing cells (Table 2). Moreover, enlarged images of the selected protein spots were used to indicate spots with significant differences between mock cells and 3CLpro-expressing cells (Fig. 2). A dramatic (greater than 100-fold) increase for Spot ID 19, a 3.3 ± 0.13-fold increase for Spot ID 55, a 5.29 ± 0.12-fold decrease for Spot ID 83, and a 6.25 ± 0.09-fold decrease for Spot ID 89 were found in 3CLpro-expressing cells (Fig. 2, Tables 1 and
Figure 1. 2-DE image for total cell extracts from human promonocyte HL-CZ cells (A) and SARS CoV 3CLpro-expressing cells (B). Protein sample (100 μg) was diluted with 350 μL of rehydration buffer, and then applied to the nonlinear Immobiline DryStrip (17 cm, pH 3–10). After incubation in the equilibration solutions, the IPG gels were transferred to the top of 12% polyacrylamide gels (20 × 20 cm × 1.0 mm). Finally, the 2-DE gels were stained with the silver nitrate solution. Protein size markers are shown at the left of each gel (in kDa). The protein spot ID numbers were consistent with those in Tables 1 and 2.
Table 1. Identification and functional classification of up-regulated proteins in SARS CoV 3CLpro-expressing cells. Biological processes associated with up-regulated proteins were categorized using Panther classification system.

| Biological process              | Spot ID | Accession | PANTHER Gene ID | Protein identification | Subcellular location | MW (KDa)/pI | Score | Peptide match | Sequence coverage (%) | Fold change | Mean SD p valuea) |
|---------------------------------|---------|-----------|-----------------|------------------------|----------------------|-------------|-------|---------------|-----------------------|-------------|-------------------|
| **Pre-mRNA processing**         |         |           |                 |                        |                      |             |       |               |                       |             |                   |
| 16                              | P07910  | 3183      |                 | Heterogeneous nuclear ribonucleoproteins C1/C2 | Nucleus              | 33.7/5.0    | 362   | 8             | 22                    | >100        | <.001             |
| 37                              | Q96AE4  | 8880      |                 | Far upstream element binding protein 1          | Nucleus              | 37.4/7.2    | 478   | 15            | 22                    | 1.9         | 0.10              | 0.002 |
| 41                              | Q99729  | 3182      |                 | Heterogeneous nuclear ribonucleoprotein A/B    | Nucleus              | 36.6/6.0    | 69    | 3             | 9                     | 10.7        | 0.36              | <.001 |
| 47                              | Q14103  | 3184      |                 | Heterogeneous nuclear ribonucleoprotein D0     | Nucleus              | 38.4/7.6    | 216   | 4             | 12                    | 3.6         | 0.08              | <.001 |
| 53                              | Q2945   | 8570      |                 | Far upstream element binding protein 2          | Nucleus              | 72.7/8.0    | 1082  | 31            | 44                    | 2.5         | 0.07              | <.001 |
| **Electron transport**          |         |           |                 |                        |                      |             |       |               |                       |             |                   |
| 1                               | P07919  | 7388      |                 | Ubiquinol-cytochrome c reductase complex 11 kDa protein | Mitochondrion        | 10.7/4.4    | 88    | 3             | 36                    | 5.6         | 0.14              | <.001 |
| 4                               | O43169  | 80777     |                 | Cytochrome b5 outer mitochondrial membrane isoform | Mitochondrion        | 163.4/8.8   | 224   | 5             | 55                    | 1.9         | 0.15              | 0.002 |
| 19                              | P06576  | 506       |                 | ATP synthase beta chain                        | Mitochondrion        | 565.5/5.3   | 973   | 19            | 42                    | >100        |                   | <.001 |
| 29                              | P10606  | 1329      |                 | Cytochrome c oxidase poly peptide Vb            | Mitochondrion        | 137.8/1.1   | 149   | 5             | 24                    | >100        |                   | <.001 |
| 31                              | P13110  | 34        |                 | Acyl-CoA dehydrogenase, medium-chain specific   | Mitochondrion        | 466.6/6.5   | 652   | 16            | 45                    | 3.8         | 0.09              | <.001 |
| 46                              | P22570  | 2232      |                 | NADPH:adrenodoxin oxidoreductase                | Mitochondrion        | 538.8/6.6   | 166   | 10            | 21                    | 2.3         | 0.14              | 0.001 |
| 49                              | P09622  | 1738      |                 | Dihydrolipoyl dehydrogenase                     | Nucleus and nucleolar| 541.7/6.5   | 342   | 10            | 22                    | 2.1         | 0.12              | 0.001 |
| 50                              | P00380  | 2396      |                 | Glutathione reductase                           | Mitochondrion and cytoplasm | 56.2/8.7   | 111   | 5             | 11                    | >100        |                   | <.001 |
| **Protein metabolism and modification** | |          |                 |                        |                      |             |       |               |                       |             |                   |
| 8                               | O43765  | 649       |                 | Small glutamine-rich tetratricopeptide repeat-containing protein A | Endoplasmic reticulum | 340.4/8.8  | 185   | 6             | 18                    | >100        |                   | <.001 |
| 11                              | P27797  | 811       |                 | Calreticulin                                     | Endoplasmic reticulum | 481.8/4.3  | 968   | 54            | 70                    | >100        |                   | <.001 |
| 16                              | P36241  | 1984      |                 | Eukaryotic translation initiation factor 5A      | Endoplasmic reticulum | 167.5/1.1  | 29    | 2             | 13                    | >100        |                   | <.001 |
| 23                              | Q15084  | 10130     |                 | Protein disulfide-isomerase A6                   | Endoplasmic reticulum | 481.8/5.5  | 389   | 5             | 15                    | >100        |                   | <.001 |
| 29                              | Q8NS9   | 81567     |                 | Thioredoxin domain-containing protein 5          | Endoplasmic reticulum | 476.5/3.2  | 259   | 7             | 16                    | >100        |                   | <.001 |
| 31                              | P17980  | 5702      |                 | 26S protease regulatory subunit 6A               | Cytoplasm and nucleus | 492.5/1.1  | 107   | 4             | 11                    | 2.8         | 0.09              | <.001 |
| 33                              | Q99471  | 5204      |                 | Prefoldin subunit 5                              | Endoplasmic reticulum | 17.3/5.9   | 82    | 1             | 11                    | 11.0        | 0.24              | <.001 |
| 27                              | P30101  | 2923      |                 | Protein disulfide-isomerase A3                   | Endoplasmic reticulum | 56.8/6.0   | 436   | 12            | 23                    | 5.9         | 0.17              | <.001 |
| 28                              | P25388  | 6206      |                 | 40S ribosomal protein S12                       | Cytoplasm             | 144.6/4.6  | 266   | 8             | 51                    | >100        |                   | <.001 |
| 30                              | P62937  | 5478      |                 | Peptidyl-prolyl cis-trans isomerase A            | Cytoplasm             | 17.9/7.8   | 312   | 7             | 40                    | 1.8         | 0.14              | 0.003 |
| 35                              | P49411  | 7284      |                 | Elongation factor Tu                            | Mitochondrion         | 493.5/7.3  | 280   | 7             | 21                    | 4.3         | 0.12              | <.001 |
| 37                              | Q96AE4  | 8880      |                 | Far upstream element binding protein 1          | Nuclear               | 674.7/2.2  | 478   | 15            | 22                    | 1.9         | 0.10              | 0.002 |
| 39                              | P20618  | 5689      |                 | Proteasome subunit beta type 1                  | Cytoplasm and nucleus | 265.8/3.3  | 125   | 4             | 19                    | 2.4         | 0.14              | 0.001 |
| 48                              | Q24752  | 38        |                 | Acetyl-CoA acetyltransferase                    | Mitochondrion         | 451.9/0.8  | 392   | 9             | 29                    | 3.1         | 0.27              | <.001 |
| 53                              | Q2945   | 8570      |                 | Far upstream element binding protein 2          | Nuclear               | 727.8/0.8  | 1082  | 31            | 44                    | 2.5         | 0.07              | <.001 |
| 57                              | P27587  | 10247     |                 | Ribonuclease UK114                              | Cytoplasm             | 145.6/7.7  | 82    | 3             | 31                    | >100        |                   | <.001 |
| 61                              | P23284  | 5479      |                 | Peptidyl-prolyl cis-trans isomerase B            | Endoplasmic reticulum | 227.8/3.3  | 100   | 3             | 14                    | 2.4         | 0.05              | 0.001 |
| 65                              | Q00688  | 2287      |                 | FK506-binding protein 3                         | Nuclear               | 25.2/9.3   | 233   | 7             | 31                    | 4.9         | 0.11              | <.001 |

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

www.proteomics-journal.com
| Biological process | Spot ID | Accession No. | Protein identification | Subcellular location | MW (KDa)/pI | Score | Peptide match | Sequence coverage (%) | Fold change Mean SD | p value<sup>a</sup> |
|--------------------|---------|---------------|------------------------|----------------------|-------------|-------|---------------|-----------------------|----------------------|-------------------|
| Nucleosome, nucleotide and nucleic acid metabolism | 6 | P83916 | 10951 | Chromobox protein homolog 1 | Cytoplasm | 21.4/4.9 | 316 | 5 | 30 | >100 | 0.001 |
| | 9 | P51858 | 3068 | Hepatoma-derived growth factor | Nucleus | 38.4/7.6 | 216 | 4 | 12 | 3.6 | 0.08 | 0.001 |
| | 10 | P06748 | 4869 | Nucleophosmin | Nucleus | 32.6/4.6 | 865 | 36 | 72 | 2.7 | 0.19 | 0.001 |
| | 16 | P07910 | 3183 | Heterogeneous nuclear ribonucleoproteins C1/C2 | Mitochondrion | 56.5/5.3 | 973 | 19 | 42 | >100 | 0.001 |
| | 25 | P15531 | 4830 | Nucleoside diphosphate kinase A | Nucleus and cytoplasm | 17.1/5.8 | 203 | 9 | 44 | >100 | 0.001 |
| | 26 | P35232 | 5245 | Prohibitin | Mitochondrion | 29.8/5.6 | 831 | 20 | 75 | >100 | 0.001 |
| | 37 | Q06AE4 | 8880 | Far upstream element binding protein 1 | Nucleus | 67.4/7.2 | 478 | 15 | 22 | 1.9 | 0.10 | 0.002 |
| | 40 | P54819 | 204 | Adenylate kinase isoenzyme 2 | Mitochondrion | 26.3/7.9 | 177 | 4 | 21 | 4.7 | 0.13 | 0.001 |
| | 41 | Q99729 | 3182 | Heterogeneous nuclear ribonucleoprotein A/B | Nucleus | 36.6/9.0 | 69 | 3 | 9 | 10.7 | 0.36 | 0.001 |
| | 47 | Q14103 | 3184 | Heterogeneous nuclear ribonucleoprotein D0 | Nucleus | 38.4/7.6 | 216 | 4 | 12 | 3.6 | 0.08 | 0.001 |
| | 51 | P34897 | 6570 | Serine hydroyxymethyltransferase | Mitochondrion | 56.0/8.8 | 661 | 17 | 35 | 2.8 | 0.10 | 0.001 |
| | 53 | Q52945 | 8570 | Far upstream element binding protein 2 | Nucleus | 72.7/8.0 | 1082 | 31 | 44 | 2.5 | 0.07 | 0.001 |
| | 54 | Q99726 | 3181 | Heterogeneous nuclear ribonucleoproteins A2/B1 | Nucleus | 37.4/9.0 | 607 | 17 | 36 | 3.5 | 0.09 | 0.001 |
| | 67 | P40926 | 4191 | Malate dehydrogenase | Mitochondrion | 35.5/9.0 | 339 | 8 | 28 | 2.1 | 0.13 | 0.001 |
| | 70 | Q95E5P | 26528 | DAZ-associated protein 1 | Cytoplasm | 43.4/8.7 | 124 | 3 | 9 | 2.7 | 0.05 | 0.001 |
| | 75 | P52722 | 4670 | Heterogeneous nuclear ribonucleoprotein M | Nucleus and nucleolar | 77.3/8.9 | 725 | 17 | 27 | 5 | 0.14 | 0.001 |
| Carbohydrate metabolism | 31 | P30084 | 1892 | Enoyl-CoA hydratase | Mitochondrion | 31.4/8.3 | 98 | 2 | 11 | 4.7 | 0.13 | 0.001 |
| | 34 | P08559 | 5160 | Pyruvate dehydrogenase E1 component alpha subunit | Mitochondrion | 43.3/8.4 | 121 | 2 | 5 | >100 | 0.001 |
| | 52 | Q16822 | 5106 | Phosphoenolpyruvate carboxykinase | Mitochondrion | 70.6/7.6 | 917 | 20 | 35 | >100 | 0.001 |
| | 54 | Q99798 | 5106 | Phosphoenolpyruvate carboxykinase | Mitochondrion | 70.6/7.6 | 917 | 20 | 35 | >100 | 0.001 |
| | 67 | P40926 | 4191 | Malate dehydrogenase | Mitochondrion | 35.5/9.0 | 339 | 8 | 28 | 2.1 | 0.13 | 0.001 |
| | 69 | P00558 | 5230 | Phosphoglycerate kinase 1 | Cytoplasm | 44.5/8.3 | 441 | 11 | 30 | >100 | 0.001 |
| Cell structure | 7 | P06753 | 7170 | Tropomyosin alpha 3 chain | Cytoplasm | 32.8/4.7 | 196 | 5 | 14 | 2.2 | 0.10 | 0.001 |
| | 17 | P63261 | 71 | Actin, cytoplasmic 2 | Cytoplasm | 41.8/5.3 | 263 | 11 | 32 | 69.6 | 2.16 | 0.001 |
| | 22 | P08670 | 7431 | Vimentin | Cytoplasm | 53.8/5.1 | 2401 | 84 | 85 | >100 | 0.001 |
| Immunity and defense | 30 | P62937 | 5478 | Peptidyl-prolyl cis-trans isomerase A | Cytoplasm | 17.9/7.8 | 312 | 7 | 40 | 1.8 | 0.14 | 0.003 |
| | 38 | P04179 | 6648 | Superoxide dismutase [Mn] | Mitochondrion | 24.7/8.4 | 87 | 2 | 9 | 2.3 | 0.09 | 0.001 |
Table 1. Continued

| Biological process | Spot ID | Accession No. | PANTHER Gene ID | Protein identification | Subcellular location | MW (KDa)/pI | Score | Peptide match | Sequence coverage (%) | Fold change | Mean SD p valuea) |
|--------------------|---------|---------------|----------------|------------------------|----------------------|-------------|-------|---------------|----------------------|-------------|-------------------|
| Amino acid biosynthesis | 61 | P23284 | 5479 | Peptidyl-prolyl cis-trans isomerase B | Endoplasmic reticulum | 22.7/9.3 | 100 | 3 | 14 | 2.4 | 0.05 | 0.001 |
| | 65 | Q00688 | 2287 | FK506-binding protein 3 | Nucleus | 25.2/9.3 | 233 | 7 | 31 | 4.9 | 0.11 | <0.001 |
| Cell cycle | 17 | P63261 | 7 | Actin, cytoplasmic 2 | Cytoplasm | 41.8/5.3 | 263 | 11 | 32 | 6.9 | 2.16 | <0.001 |
| | 26 | P35232 | 5245 | Prohibitin | Mitochondrion | 28.8/5.6 | 831 | 20 | 75 | >100 | <0.001 |
| | 65 | Q00688 | 2287 | FK506-binding protein 3 | Nucleus | 25.2/9.3 | 233 | 7 | 31 | 4.9 | 0.11 | <0.001 |
| Amino acid metabolism | 42 | P13995 | 10797 | Methylene-tetrahydrofolate dehydrogenase/cyclohydrolase | Mitochondrion | 37.3/8.9 | 173 | 4 | 16 | 2.4 | 0.15 | 0.001 |
| | 44 | Q9Y617 | 29968 | Phosphoserine aminotransferase | Mitochondrion | 40.4/7.6 | 394 | 10 | 29 | >100 | <0.001 |
| Apoptosis | 55 | O95831 | 9131 | Apoptosis-inducing factor | Mitochondrion | 66.9/9.0 | 252 | 8 | 18 | 3.3 | 0.13 | <0.001 |
| Sulfur redox metabolism | 5 | O00264 | 10857 | Membrane-associated progesterone receptor component 1 | Microsome | 21.5/4.6 | 172 | 5 | 20 | 3.8 | 0.18 | <0.001 |
| Anion transport | 66 | P21796 | 7416 | Voltage-dependent anion-selective channel protein 1 | Mitochondrion | 30.6/8.6 | 255 | 5 | 25 | >100 | <0.001 |
| Steroid hormone-mediated signaling | 14 | Q95881 | 51060 | Thioredoxin-domain-containing protein 12 | Endoplasmic reticulum | 19.2/5.2 | 191 | 6 | 57 | 12.7 | 0.14 | <0.001 |
| Muscle contraction | 15 | P12829 | 4635 | Myosin light polypeptide 4 | Mitochondrion | 21.4/5.0 | 411 | 10 | 46 | 3.4 | 0.09 | <0.001 |
| Unclassified | 2 | Q9UMS0 | 27247 | HIRA-interacting protein 5 | Mitochondrion | 21.8/4.2 | 286 | 6 | 33 | 2.1 | 0.16 | <0.001 |
| | 3 | P20674 | 9377 | Cytochrome C oxidase polypeptide Va | Mitochondrion | 16.8/6.3 | 267 | 8 | 46 | >100 | <0.001 |
| | 32 | P13804 | 2108 | Electron transfer flavoprotein alpha-subunit | Mitochondrion | 35.1/8.6 | 763 | 15 | 56 | 1.6 | 0.21 | 0.005 |
| | 56 | P07737 | 5216 | Profilin-1 | Mitochondrion | 14.9/8.5 | 355 | 10 | 81 | 65.8 | 1.68 | <0.001 |
| | 58 | Q9NPJ3 | 55856 | Thioredoxin superfamily member 2 | Mitochondrion | 15.0/9.2 | 87 | 2 | 17 | >100 | <0.001 |
| | 59 | P62807 | 8339 | Histone H2B.a/g/h/k/l | Nucleus | 13.8/10.3 | 211 | 6 | 40 | >100 | <0.001 |
| | 63 | Q9Y203 | 373156 | Glutathione S-transferase kappa 1 | Peroxisome | 25.3/8.5 | 189 | 3 | 20 | 5.9 | 0.24 | <0.001 |
| | 73 | P25705 | 498 | ATP synthase alpha chain | Mitochondrion | 59.7/9.2 | 784 | 14 | 34 | >100 | <0.001 |

a) Student's t-test.
Figure 2. Close-up comparisons of spots on 2-DE images. The interested protein spots showing significant expression differences were enlarged. The circles indicated protein spots of total cell extracts from mock cells (left) and 3CLpro-expressing cells (right). The protein spot ID numbers were consistent with those in Tables 1 and 2.

2). These selected protein spots were picked out of the stained gel, subjected to in-gel tryptic digestion, and underwent PMF using the NanoLC Trap Q-TOF MS (Tables 1 and 2). The representative peptide peaks from Q-TOF MS analysis were detected, such as 26S protease regulatory subunit 6A (Spot ID 21) (Fig. 3A) and apoptosis-inducing factor (Spot ID 55) (Fig. 3B), resulting in confident protein identification by MASCOT searching. The search results indicated that 73 up-regulated and 21 down-regulated proteins showed the best match with a protein score of greater than or equal to 67, considered to be significant using the MASCOT search algorithm (p<0.05) (Tables 1 and 2). The amino acid sequence coverage of identified up-regulatory and down-regulatory proteins varied from 9 to 85%. For example, ubiquitin-conjugating enzyme E2 N (Spot ID 24) had a MASCOT score of 217, sequence coverage of 52%, and eight matched peptides, while apoptosis-inducing factor (Spot ID 55) showed a MASCOT score of 252, sequence coverage of 18%, and eight matched peptides. Therefore, comparative analysis of protein profiling indicated that 73 up-regulated and 21 down-regulated proteins were identified in 3CLpro-expressing cells.

3.2 Functional classification of the identified up-regulated and down-regulated proteins

As for the implication of cellular responses to SARS CoV 3CLpro, these up-regulated and down-regulated proteins were further categorized according to their subcellular location, biological process and biological pathway using the PANTHER classification system (Figs. 4 and 5, Tables 1, 2 and 3). Interestingly, up-regulated proteins in 3CLpro-expressing cells were mainly located in the mitochondrion (26/73, 36%) (Fig. 4A). By contrast, down-regulated proteins were distributed within different parts of the cells (19% in mitochondrion, 24% in cytoplasm, and 10% in nucleus) (Fig. 4B). Biological process categorization revealed a diversity of biological processes associated with the proteins identified (Fig. 5). The up-regulated proteins were responsible for the five main biological processes of protein metabolism and modification, nucleoside, nucleotide and nucleic acid metabolism, electron transport, pre-mRNA processing, and immunity and defense (Fig. 5, Table 1). Comparison of the sub-categories of protein metabolism and modification showed significant differences between the biological process of up-regulated and down-regulated proteins (Fig. 5). The biological processes of proteolysis and protein modification were significantly up-regulated, but the biological processes of the protein biosynthesis and protein complex assembly were down-regulated in 3CLpro-expressing cells. Furthermore, 26S protease regulatory subunit 6A (Spot ID 21) and ubiquitin-conjugating enzyme E2 N (Spot ID 24), which is up-regulated in the biological processes of proteolysis and protein modification that are key to the ubiquitin proteasome pathway (Table 3). According to the biological pathway categorization, up-regulated proteins are associated with 11 signaling pathways, including de novo purine biosynthesis, ubiquitin proteasome, ATP synthesis, and apoptosis signaling pathways (Table 3). Identified down-regulatory proteins in 3CLpro-expressing cells were involved in five signaling pathways, including de novo purine biosynthesis, apoptosis signaling, and mRNA splicing pathways (Table 3). Interestingly, analysis of apoptosis signaling pathway revealed that the mitochondrial apoptogenic apoptosis-inducing factor (Spot ID 55) was up-regulated and anti-apoptogenic heat shock cognate 71-kDa protein (HSP70) (Spot ID 83) was down-regulated in 3CLpro-expressing cells (Table 3). This finding suggested that expression of SARS CoV 3CLpro resulted in activation of the apoptosis signaling pathway.

3.3 Expression increases of 26S protease regulatory subunit 6A and apoptosis-inducing factor

To confirm the expression levels of these identified proteins, Western blotting analysis of cell lysates from mock cells and SARS-CoV 3CLpro-expressing cells was carried out, in which beta-actin was used as an internal control (Fig. 6). After normalization with beta-actin, densitometric analysis of immuno-
reactive bands revealed that 26S protease regulatory subunit 6A and apoptosis-inducing factor were significantly increased 3- and 1.5-fold, respectively, in 3CLpro-expressing cells. The results were consistent with proteomic analyses of silver-stained 2-DE gels as shown in Fig. 1.

3.4 Subcellular localization of apoptosis-inducing factor and cytochrome c

To further investigate the role of mitochondria in SARS 3CLpro-induced apoptosis, confocal imaging analysis was applied to determine subcellular localization of apoptosis-inducing factor (Fig. 7). HL-CZ cells were transiently co-transfected with pcDNA3.1 or p3CLpro plus a mitochondrial localization vector pDsRed-Mito. After immunofluorescent staining, apoptosis-inducing factor labeled with FITC-conjugated secondary antibodies showed green fluorescence, whereas mitochondria were targeted by red fluorescent proteins (Fig. 7A). Confocal imaging of the stained cells revealed that the release of apoptosis-inducing factor from mitochondria was found in the SARS CoV 3CLpro-expressing cells (Fig. 7A, right), but not in mock cells (Fig. 7A, left). In addition, cytochrome c, the other mitochondrial pro-apoptotic protein, was also found to be released from mitochondria to cytosol in 3CLpro-expressing cells (Fig. 7B). The results indicated that SARS CoV 3CLpro induced mitochondria
Table 2. Identification and functional classification of down-regulated proteins in SARS CoV 3CLpro-expressing cells. Biological processes associated with down-regulated proteins were classified using Panther Classification system

| Biological process | Spot No. | Accession No. | PANTHER Gene ID | Protein identification | Subcellular location | MW (KDa)/pI | Score | Peptide match | Sequence coverage (%) | Fold change Mean/SD | p valuea) |
|--------------------|----------|---------------|-----------------|-----------------------|----------------------|-------------|-------|--------------|----------------------|-------------------|-----------|
| Protein metabolism and modification | 76 | P05386 | 6176 | 60S acidic ribosomal protein P1 | | 11.5/4.26 | 152 | 2 | 51 | 2.89 | 0.09 | <0.001 |
| | 77 | P05387 | 6181 | 60S acidic ribosomal protein P2 | | 11.7/4.42 | 385 | 7 | 77 | 4.27 | 0.13 | <0.001 |
| | 78 | P24534 | 1933 | Elongation factor 1-beta | | 24.6/4.5 | 367 | 10 | 37 | 2.45 | 0.25 | 0.007 |
| | 80 | P07237 | 5034 | Protein disulfide-isomerase precursor | Endoplasmic reticulum | 57.1/4.76 | 1097 | 30 | 51 | 5.73 | 0.31 | <0.001 |
| | 82 | P10809 | 3329 | 60 kDa heat shock protein | Mitochondrion | 61.0/5.7 | 1404 | 39 | 43 | 2.27 | 0.19 | 0.005 |
| | 83 | P11142 | 3312 | Heat shock cognate 71 kDa protein | Cytoplasm | 70.8/5.37 | 1287 | 31 | 47 | 5.29 | 0.12 | <0.001 |
| | 84 | Q13347 | 8668 | Eukaryotic translation initiation factor 3 subunit 2 | | 36.5/5.38 | 334 | 6 | 19 | 3.89 | 0.39 | <0.001 |
| | 85 | Q13347 | 8668 | Eukaryotic translation initiation factor 3 subunit 2 | | 36.5/5.38 | 334 | 6 | 19 | 3.89 | 0.39 | <0.001 |
| | 86 | P31948 | 10903 | Stress-induced-phosphoprotein 1 | Cytoplasm and nucleus | 62.6/6.4 | 1091 | 30 | 46 | 4.1 | 0.09 | <0.001 |
| | 88 | Q9UMS4 | 27339 | Pre-mRNA-splicing factor 19 | Nucleus | 55.1/6.14 | 357 | 10 | 22 | 3.24 | 0.11 | <0.001 |
| Nucleoside, nucleotide and nucleic acid metabolism | 93 | P20290 | 689 | Transcription factor BTF3 | Nucleus | 22.1/9.41 | 317 | 7 | 53 | 2.54 | 0.19 | 0.002 |
| Cell structure and motility | 81 | P52907 | 829 | F-actin capping protein alpha-1 subunit | | 32.9/5.45 | 486 | 11 | 53 | 3.52 | 0.28 | <0.001 |
| | 89 | P04083 | 301 | Annexin A1 | | 39.8/6.64 | 1299 | 22 | 63 | 7.03 | 0.15 | <0.001 |
| Immunity and defense | 79 | Q07021 | 708 | Complement component 1, Q subcomponent-binding protein | Mitochondrion | 31.3/4.74 | 321 | 5 | 21 | 8.59 | 0.3 | <0.001 |
| | 83 | P11142 | 3312 | Heat shock cognate 71 kDa protein | Cytoplasm | 70.8/5.37 | 1287 | 31 | 47 | 5.29 | 0.12 | <0.001 |
| Fatty acid biosynthesis | 94 | Q04828 | 1645 | Aldo-keto reductase family 1 member C1 | Cytoplasm | 36.8/6.02 | 137 | 7 | 25 | 2.15 | 0.12 | 0.007 |
| Other carbon metabolism | 86 | P05091 | 217 | Alddehyde dehydrogenase | Mitochondrion | 56.3/6.63 | 110 | 4 | 6 | 3.13 | 0.06 | <0.001 |
| | 91 | P00352 | 216 | Retinal dehydrogenase | Cytoplasm | 54.7/6.29 | 436 | 11 | 24 | 2.71 | 0.16 | 0.002 |
| Unclassified | 85 | P08650 | 56945 | Mitochondrial 28S ribosomal protein S22 | Mitochondrion | 41.2/7.7 | 466 | 14 | 31 | 3.54 | 0.2 | <0.001 |
| | 95 | P08805 | 3921 | 40S ribosomal protein SA (p40) | (34/67 kDa laminin receptor) | Cytoplasm | 32.7/4.79 | 512 | 12 | 43 | 6.65 | 0.31 | <0.001 |

a) Student's t-test.
alternations in release of apoptosis-inducing factor and cytochrome c, therefore responsible for activation of upstream caspase-9 and downstream caspase-3.

4 Discussion

In this study, 73 up-regulated and 21 down-regulated proteins in 3CLpro-expressing cells were identified using the combined analysis of 2-DE and Q-TOF MS (Figs. 1 and 2, Tables 1 and 2). Of the up-regulated proteins identified, 36% (26/73) were mitochondrial proteins that are associated with many biological processes, particularly in electron transport, ATP synthesis, carbohydrate metabolism and apoptosis (Fig. 4A, Table 1). By contrast, only 19% of down-regulated proteins were located within mitochondrion (Fig. 4B). Up-regulation of activation of the mitochondrial electron transport system coupled with ATP synthesis was identified in SARS 3CLpro-expressing cells (Fig. 5, Tables 1 and 3), in which ATP synthase (Spot ID 19) and cytochrome c oxidase were also involved in the control of mitochondrial membrane potential $\Delta \Psi m$ and formation of reactive oxygen species (ROS) [31]. This finding could be associated with generation of ROS in 3CLpro-expressing cells as described in our previous report [16].

Protein metabolism and modification was the major biological process for up-regulated and down-regulated proteins in 3CLpro-expressing cells (Fig. 5). However, analysis of the biological process indicated that proteolysis and protein modification were significantly up-regulated, but protein biosynthesis and protein complex assembly were down-regulated in 3CLpro-expressing cells. Significant increases of
Figure 5. Comparison of biological processes associated with up-regulated and down-regulated proteins in 3CLpro-expressing cells compared with mock cells. Biological processes associated with up- and down-regulated proteins were classified using Panther Classification system (http://www.pantherdb.org/). Percent of biological process was calculated as the number of identified proteins in the indicated biological process/the number of the total identified proteins × 100.

Table 3. Comparison of biological pathways associated with up-regulated and down-regulated proteins in 3CLpro-expressing cells. Biological pathways associated with proteins identified were classified using Panther Classification system

| Pathways                                      | Spot ID | Protein identification                | Pathways                                      | Spot ID | Protein identification                |
|-----------------------------------------------|---------|---------------------------------------|-----------------------------------------------|---------|---------------------------------------|
| De novo purine biosynthesis                   | 25      | Nucleoside diphosphate kinase A       | De novo purine biosynthesis                   | 89      | Bifunctional purine biosynthesis protein PURH |
|                                               | 40      | Adenylate kinase isoenzyme 2          |                                               |         |                                       |
|                                               | 64      | GTP:AMP phosphotransferase mitochondrial |                                               |         |                                       |
| Apoptosis signaling                           | 55      | Apoptosis-inducing factor              | Apoptosis signaling                           | 83      | Heat shock cognate 71 kDa protein     |
| Ubiquitin proteasome                          | 21      | 26S protease regulatory subunit 6A     | Ubiquitin proteasome                          | 24      | Phenylethylamine degradation          |
|                                               | 24      | Ubiquitin-conjugating enzyme E2 N     |                                               |         | Aldehyde dehydrogenase               |
| ATP synthesis                                 | 19      | ATP synthase beta chain               | mRNA splicing                                 | 88      | Pre-mRNA-splicing factor 19           |
|                                               |         |                                      | 5-Hydroxytryptamine degradation               | 86      | Aldehyde dehydrogenase               |
| Glutamine glutamate conversion                | 36      | Glutamate dehydrogenase 1             |                                               | 91      | Retinal dehydrogenase 1              |
| Asparagine and aspartate biosynthesis         | 72      | Aspartate aminotransferase            |                                               |         |                                       |
| TCA cycle                                     | 45      | Fumarate hydratase                    |                                               |         |                                       |
| Glycolysis                                    | 69      | Phosphoglycerate kinase 1             |                                               |         |                                       |
|                                               | 62      | Histone deacetylase complex subunit SAP18 |                                               |         |                                       |
| Toll receptor signaling pathway               | 24      | Ubiquitin-conjugating enzyme E2 N     | 5-Hydroxytryptamine degradation               | 86      | Aldehyde dehydrogenase               |
|                                               |         |                                      |                                               | 91      | Retinal dehydrogenase 1              |
| Cytoskeletal regulation by Rho GTPase         | 56      | Profilin-1                            |                                               |         |                                       |
the 26S protease regulatory subunit 6A (Spot ID 21) and ubiquitin-conjugating enzyme E2 N (Spot ID 24), which is involved in the ubiquitin proteasome pathway was demonstrated by silver staining of 2-DE gels and Western blotting (Figs. 1, 2 and 6). Interestingly, up-regulation of proteasome subunits was also found in SARS CoV-infected Vero E6 cells and hepatitis virus B (HBV) HBx-expressing mice [20, 22]. The ubiquitin-proteasome pathway plays a central role in several cellular processes including antigen processing, apoptosis, cell cycle, inflammation, and response to stress [32], and is involved in the replication of several viruses, such as mouse hepatitis virus (murine coronavirus) [33, 34], adenovirus [35], hepatitis C virus [36], and human immunodeficiency virus [37]. Therefore, up-regulation of the ubiquitin-proteasome pathway induced by SARS-CoV 3CLpro protein might be involved in the SARS pathogenesis.

Five kinds (C1/C2, A/B, D0, A2/B1 and M) of heterogeneous nuclear ribonucleoproteins (hnRNP) were identified as being significantly up-regulated in SARS-CoV 3CLpro-expressing cells (Fig. 1, Table 1), and responsible for transcription, pre-mRNA processing, mRNA splicing, and nucleoside, nucleotide and nucleic acid metabolism (Fig. 5 and Table 1). The finding was in agreement with the protein profiling of SARS-CoV-infected cells in a previous report [20]. HnRNP have also been reported to be involved in the replication of mouse hepatitis virus (MHV) [38, 39]. In addition, hnRNP A2/B1 and A/B have been demonstrated to interact with the negative-strand MHV leader RNA and to enhance

![Figure 6](image-url)

Figure 6. Western blot analysis of 26S protease regulatory subunit 6A and apoptosis-inducing factor in mock cells and 3CLpro-expressing cells. Each lysate was analyzed by 12% SDS-PAGE, and then electrophoretically transferred onto NC paper. The blot was probed with monoclonal antibodies to 26S protease regulatory subunit 6A and apoptosis-inducing factor, and developed with an alkaline phosphatase-conjugated secondary antibody and NBT/BCIP substrates. Lane 1: mock cells; lanes 2: 3CLpro-expressing cells.

![Figure 7](image-url)

Figure 7. Confocal image analysis of apoptosis-inducing factor (A) and cytochrome c (B) in mock cells and 3CLpro-expressing cells. HL-CZ cells were transiently co-transfected with plasmid pcDNA3.1 or pSARS-CoV 3CLpro plus a mitochondrial localization vector pDsRed-Mito (enhanced red fluorescent protein). After immunofluorescent staining, apoptosis-inducing factor and cytochrome c were probed by FITC-conjugated secondary antibodies. Confocal image analysis of the cells was performed using Leica TCS SP2 AOBS laser-scanning microscopy.
We would like to thank China Medical University and National Science Council, Taiwan for financial supports (CMU95-152, CMU95-153, NSC94-2320-B-039-010 and NSC95-2320-B-039-019).

5 References

[1] Lee, N., Hui, D., Wu, A., Chan, P. et al., N. Engl. J. Med. 2003, 348, 1986–1994.
[2] Tsang, K. W., Ho, P. L., Ooi, G. C., Yee, W. K. et al., N. Engl. J. Med. 2003, 348, 1977–1985.
[3] Hsueh, P. R., Chen, P. J., Hsiao, C. H., Yeh, S. H. et al., Emerg. Infect. Dis. 2004, 10, 489–493.
[4] Nicholls, J. M., Poon, L. L., Lee, K. C., Ng, W. F. et al., Lancet 2003, 361, 1773–1778.
[5] Lang, Z., Zhang, L., Zhang, S., Meng, X. et al., Chin Med J (Engl). 2003, 116, 976–980.

© 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
[35] Galinier, R., Gout, E., Lortat-Jacob, H., Wood, J. et al., Biochemistry 2002, 41, 14299–14305.
[36] Gao, L., Tu, H., Shi, S. T., Lee, K. J. et al., J. Virol. 2003, 77, 4149–4159.
[37] Schubert, U., Ott, D. E., Chertova, E. N., Welker, R. et al., Proc. Natl. Acad. Sci. USA 2000, 97, 13057–13062.
[38] Li, H. P., Zhang, X., Duncan, R., Comai, L. et al., Proc. Natl. Acad. Sci. USA 1997, 94, 9544–9549.
[39] Li, H. P., Huang, P., Park, S., Lai, M. M., J. Virol. 1999, 73, 772–777.
[40] Shi, S. T., Yu, G. Y., Lai, M. M., J. Virol. 2003, 77, 10584–10593.
[41] Joza, N., Susin, S. A., Daugas, E., Stanford, W. L. et al., Nature 2001, 410, 549–554.
[42] Yu, S.W., Wang, H., Poitras, M. F., Coombs, C. et al., Science 2002, 297, 259–263.