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Expression and functional characterisation of the putative SARS coronavirus non-structural proteins X1-X5

Key Messages

1. We produced mammalian expression vectors encoding the SARS coronavirus (SARS-CoV) accessory proteins with or without the fluorescence protein tag and cell lines with stable expression of these proteins.
2. The cellular localisation and function of the SARS-CoV accessory proteins was determined.
3. SARS 6 and SARS 8b proteins are localised to the endoplasmic reticulum and nucleus/cytoplasm, respectively, and both proteins stimulate host cell DNA synthesis.

Introduction

With the SARS coronavirus (SARS-CoV) genome information in place, the challenge of fighting the viral infection and its complications depends on a comprehensive understanding of how the components of the viral genome coordinate the infection and replication events. The problems of using the whole virus for experiments are the risks of infection and the difficulties segregating the functions of different effectors. It is important to express the viral proteins in vitro for further characterisation using recombinant DNA technology.

The SARS-CoV genome encodes 14 predicted open reading frames (Orfs) and synthesises eight subgenomic mRNAs. Five putative accessory proteins of 50 amino acids or larger are the products of Orfs 3a, 3b, 6, 7a, and 8b, producing the putative accessory proteins SARS 3a, 3b, 6, 7a, and 8b (also known as X1, X2, X3, X4, and X5), accordingly.[1] Research efforts have been focused on characterising the viral structural proteins and enzymes. Major knowledge gaps exist concerning the biological significance of the expression of the accessory proteins.

It is generally recognised that the structural proteins contribute to the virulence and immunogenicity of a virus. However, the importance of viral accessory proteins in the viral life cycle should not be overlooked. Interestingly, induction of cell proliferation and apoptosis are involved in coronavirus infection. Coronavirus infection in the early stages had been reported to stimulate epithelial cells, causing cell proliferation and squamous metaplasia in the lungs.[2] Examination of pathological tissues of SARS patients revealed significantly increased cell apoptotic activities in spleen, lung, and lymph nodes as compared to the normal tissues.[3] The effects of SARS-CoV accessory proteins on cell proliferation and survival responses were investigated in this project.

Aims and objectives

The aim of this project was to develop research tools and use them to characterise the roles of SARS-CoV accessory proteins in the growth and survival of cells representing the pathological sites of SARS. The ultimate goal was to facilitate better understanding of the SARS-CoV biology. The objectives of the study were to:

1. construct mammalian expression vectors for the SARS-CoV genes;
2. study cellular localisation of the SARS-CoV accessory proteins; and
3. characterise the effects of SARS-CoV accessory proteins on cell proliferation and viability.

Methods

This study was conducted from January 2005 to December 2005.

Setting and subjects

CHO and Vero E6 cells of epithelial origin were used as model systems. The cDNAs encoding the SARS-CoV accessory proteins were first cloned by

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polymerase chain reaction (PCR) with DNA sequences confirmed. Subsequently, the cDNAs were subcloned into mammalian expression vectors with or without the green fluorescence protein (GFP) or red fluorescence protein (RFP) fusion.

Study instruments

For the DNA cloning, PCR-cloned viral cDNA fragments were subcloned into pEGFP-N1, pDsRed-N1, or pcDNA3.1 vectors as described elsewhere.[4,5] DNA ligation, transformation/selection/propagation in *Escherichia coli*, and purification of the plasmids were performed according to standard protocols. DNA sequencing analyses were performed to confirm the correct identity and orientation of the subcloned fragments.

For the cell culture, cell lines were maintained at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. For expression of cloned viral genes, constructs encoding the recombinant viral proteins were introduced into cells by the Lipofectamine 2000 Transfection Reagent (Invitrogen Corp, Carlsbad, CA, US) as per the manufacturer's instructions.

Interventions

Group A constructs were designed for cellular localisation studies. Mammalian pEGFP-N1 and pDsRed-N1 vectors (BD Biosciences, Clontech, Palo Alto, CA, US) were used to generate plasmids encoding fusion proteins with GFP or RFP tagged to the C terminus of each of the SARS-CoV accessory proteins. Group B constructs were designed for cell proliferation and viability studies. The cDNAs encoding the SARS-CoV accessory proteins were individually cloned into the pcDNA3.1 vectors (Invitrogen Corp). No reporter tag was fused to the encoded proteins.

Main outcome measures

Group A constructs were transfected into cells for cellular localisation studies by confocal microscopy. Cells transfected with the Group A constructs also served as a tool to provide preliminary data for the effects of SARS-CoV accessory protein expression on cell function. Group B constructs were transfected into cells for transient or stable expression. The expression of these constructs allows for the evaluation of the effects of the SARS-CoV accessory proteins on cell proliferation and viability without the potential interference of a protein tag. Cells expressing the native SARS-CoV accessory proteins were subjected to cell growth and viability analyses using the ^{3}H-thymidine incorporation assay and flow cytometry.

Results

We proposed to characterise five accessory proteins with full awareness that the SARS-CoV related studies are competitive and time-sensitive. Not surprisingly, the reports for SARS 3a, 3b, and 7a were published during the project period. We therefore focused our research effort on characterising the SARS 6 and 8b proteins.

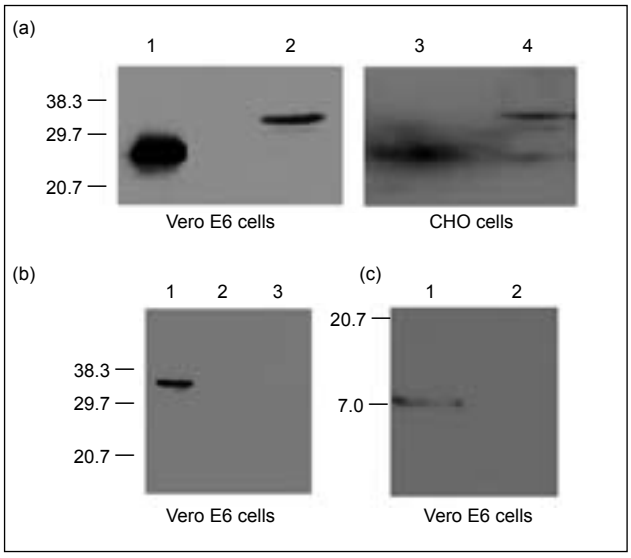


Fig 1. Expression of recombinant SARS 6 protein
(a) Lanes 1, 3: EGFP; Lanes 2, 4: SARS 6-EGFP fusion. (b) Lane 1: SARS 6-EGFP fusion. (c) Lane 1: native SARS-6

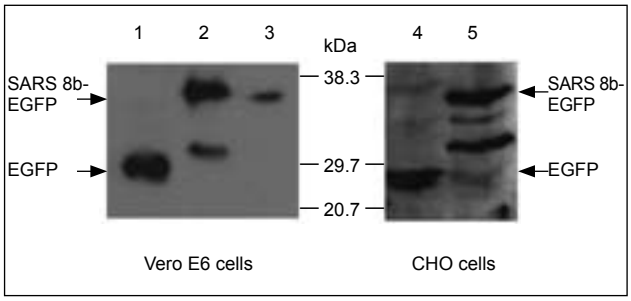


Fig 2. Expression of recombinant SARS 8b-EGFP protein
Lanes 1, 4: EGFP; Lanes 2, 3, 5: SARS 8b-EGFP fusion

Expression of recombinant SARS-CoV accessory proteins

The expression of the SARS 6-EGFP fusion protein was determined by Western blotting using anti-GFP or anti-SARS 6 primary antibodies (Fig 1a, 1b). In both Vero E6 and CHO cells, the anti-GFP antibody recognised the expressed SARS 6-EGFP fusion protein showing the anticipated ~34.5 kDa band (Fig 1a, Lanes 2, 4). The anti-SARS 6 antibody also recognised a single band with the expected molecular size of the SARS 6-GFP fusion protein (Fig 1b, Lane 1). Using the same antibody, expression of the untagged SARS 6 protein in Vero E6 cells was also confirmed (Fig 1c, Lane 1).

The expression of the EGFP and SARS 8b-EGFP proteins in CHO and Vero E6 cells was detected by Western blotting using anti-GFP primary antibodies (Fig 2). The expected bands of EGFP (~27 kDa) and SARS 8b-EGFP

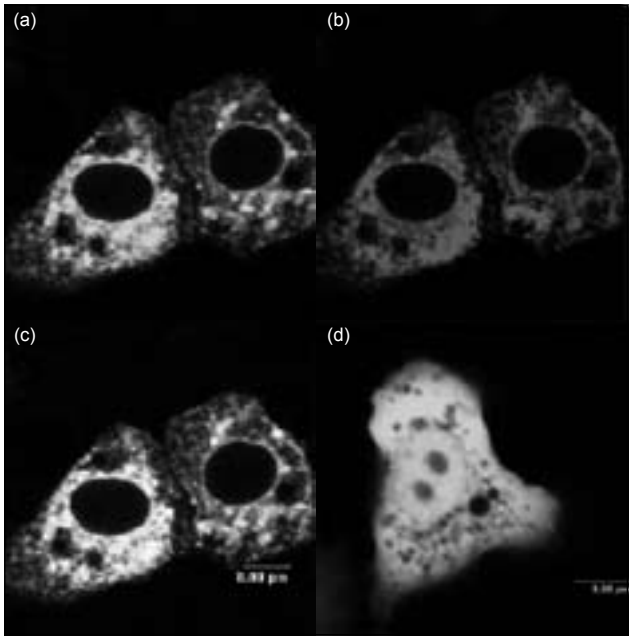


Fig 3. Subcellular localisation of recombinant SARS 6 protein in Vero E6 cells
(a) SARS 6-EGFP fusion; (b) ER marker; (c) superimposed images of (a) and (b); (d) EGFP

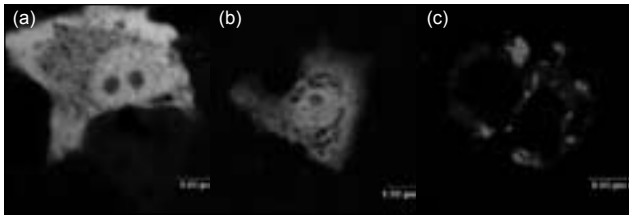


Fig 4. SARS 8b-GFP and SARS 6-RFP protein expression in Vero E6 cells
(a) EGFP; (b) SARS 8b-EGFP; (C) SARS 6-RFP

(~36.6 kDa) were observed, indicating the proteins were expressed in the cells.

Subcellular localisation of SARS-CoV accessory proteins

The subcellular localisation of the SARS 6 protein in mammalian cells was determined by expressing the SARS 6-EGFP fusion constructs in Vero E6 and CHO cells. Confocal microscopy showed that SARS 6-EGFP protein was localised to the perinuclear region and colocalised with the ER marker (Fig 3a-c) in Vero E6 cells. A similar localisation pattern of the SARS 6-EGFP was obtained in CHO cells (data not shown). In contrast, the control EGFP protein was distributed throughout the cells as expected (Fig 3d).

The SARS 8b-EGFP fusion protein was used in this study for the cellular localisation of SARS 8b analysis to circumvent the problem of the lack of SARS 8b antibodies. Results of confocal microscopy showed that the fusion SARS 8b-EGFP protein possessed a similar fluorescent pattern as the EGFP control, because the signals for both proteins were observed in the cytosol and nuclei in Vero E6 cells (Fig 4a, b). The SARS 6-RFP fusion protein results further substantiated its ER localisation (Fig 4c) as described above. A similar localisation pattern of the SARS 8b-EGFP was obtained in CHO cells (data not shown). The possibility that SARS 6 and SARS 8b viral accessory proteins interact and redistribute in the cell was investigated. Confocal microscopy results indicated that the co-expression of these two proteins do not incur observable changes of localisation, relative to individually expressed SARS 6 or SARS 8b (data not shown). However, this observation does not completely rule out the possibility of interaction between the two proteins.

Thymidine incorporation studies

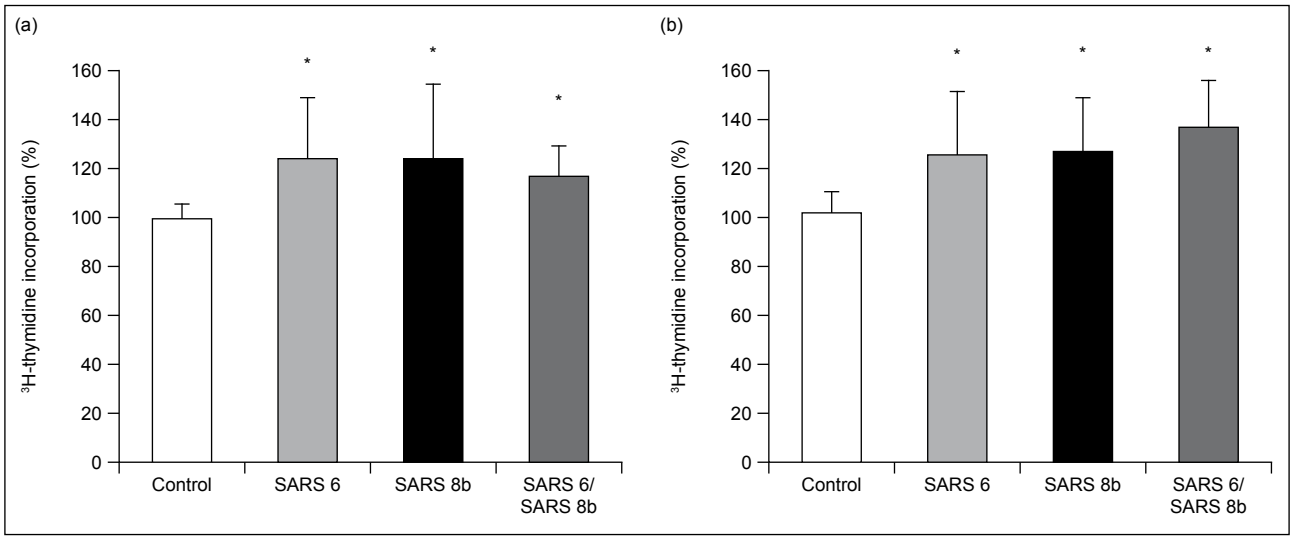
Coronavirus infection in the early stages had been reported to stimulate epithelial cells, causing cellular proliferation and squamous metaplasia in the lungs.[2] In an attempt to elucidate the biological function of the SARS 6 and SARS 8b proteins, [^{3}H]-thymidine incorporation was measured in both CHO and Vero E6 cells expressing the untagged SARS 6 and/or SARS 8b proteins so as to evaluate their effects on DNA synthesis. Real-time PCR confirmed the expression of the mRNA of these untagged SARS-CoV genes in both Vero E6 and CHO cells (data not shown). Figure 5 shows that individual expression of the SARS 6 protein induced DNA synthesis in CHO cells and in Vero E6 cells. Furthermore, co-expression experiments indicated that the untagged SARS 6 and SARS 8b proteins in the cells do not elicit additional or synergistic effects on DNA synthesis.

Discussion

The cell proliferative effect of SARS 6 and 8b in epithelial cell lines used in this study was consistent with the pathology of epithelial proliferation observed in SARS patients.[2] Currently, the significance of SARS 6 and SARS 8b induced DNA synthesis in SARS-CoV biology remains elusive, since our observations were obtained independently of the whole viral genome. Nevertheless, such biological activities further substantiate the expression of the SARS 6 and SARS 8b proteins in the cells, and imply their interaction with cellular components.

Since both SARS 6 and 8b stimulate DNA synthesis, it is conceivable that these proteins do not elicit apoptotic effects. Such a notion is supported by flow cytometry analyses showing that expression of these proteins did not lead to apoptotic events in the host cells (data not shown). More comprehensive results are presented in published reports.[4,5]

To conclude, the research tools and materials produced in this project (mammalian expression vectors of the SARS-CoV accessory proteins and cell lines with stable



* Significant differences among the controls and SARS proteins–expressing cells detected by Kruskal-Wall ANOVA on Ranks test ($P<0.05$)

Fig 5. Stimulation of DNA synthesis by untagged SARS 6 and SARS 8b proteins
(a) Vero E6 cells; (b) CHO cells

expression of these proteins) facilitated our goal, which was to advance knowledge on the function of SARS-CoV accessory proteins. The cloning and expression of the SARS-CoV accessory viral proteins fused to the fluorescent reporter protein circumvented the problem posed by lack of antibodies against these proteins, and revealed their cellular localisation. The research tools and materials as well as the knowledge this project has yielded will facilitate future investigation towards the understanding of the interactive effects of the SARS-CoV genome components.

Acknowledgements

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Results of this study were published in full in:

(1) FEBS Letters: Geng H, Liu YM, Chan WS, et al. The putative protein 6 of the severe acute respiratory syndrome-associated coronavirus: expression and functional characterization. FEBS Lett 2005;579:6763-8.

(2) FEBS Letters: Law PY, Liu YM, Geng H, Kwan KH, Waye MM, Ho YY. Expression and functional characterization of the putative protein 8b of the severe acute respiratory syndrome-associated coronavirus. FEBS Lett 2006;580:3643-8.

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