

# Modulation of cell signalling by human coronavirus HKU1 S and M proteins

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## KEY MESSAGES

1. S proteins of both SARS-CoV and HCoV-HKU1 share a similar profile of unfolded protein response (UPR)-activating activity. They activate the production of UPR effector proteins Grp78/94 and to a lesser extent CHOP through PERK kinase.
2. S proteins of SARS-CoV and HCoV-HKU1 have distinct UPR-activating domains. Whereas the UPR-activating activity requires the central region (amino acids 201-400) of the S1 subunit in SARS-CoV, the corresponding part in the HCoV-HKU1 S protein does not induce endoplasmic reticulum stress or UPR.
3. Suppression of type I interferon (IFN) production is a unique property of SARS-CoV M protein. HCoV-HKU1 M protein does not inhibit the

innate IFN response.

4. IFN antagonism of SARS-CoV M protein is ascribed to its first transmembrane domain (TM1) situated at the N terminus. TM1 targets M protein to the Golgi complex and interacts with TRAF3 and other transducer proteins to prevent the formation of a functional TRAF3-containing multi-component complex.

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## Introduction

In 2003, an outbreak of severe acute respiratory syndrome (SARS) occurred in Hong Kong and spread worldwide. The SARS coronavirus (SARS-CoV) caused a highly lethal disease and disproved the concept that human coronaviruses are generally associated with mild respiratory disease. Another new coronavirus—HCoV-HKU1—that circulates commonly in the human population and causes relatively milder respiratory tract illness worldwide was also discovered.<sup>1</sup> The mechanism by which SARS-CoV and HCoV-HKU1 cause respiratory diseases of different severity remains elusive. Variations in their ability to modulate cell signalling and innate immunity might be influential.<sup>2</sup>

Human coronaviruses are enveloped and positive-stranded RNA viruses with a large genome. They hijack the endoplasmic reticulum (ER) to process their structural and non-structural proteins produced in extraordinarily large amounts. They also need to circumvent the production and function of type I interferons (IFNs), major effectors of innate antiviral immunity. We have demonstrated the capability of SARS-CoV S protein to induce ER stress and to activate the unfolded protein response (UPR).<sup>3</sup> We have also found that SARS-CoV M protein suppresses innate IFN production by impeding the formation of a functional complex of tumour necrosis factor receptor-associated factor 3 (TRAF3) - TRAF family member-associated NF- $\kappa$ B activator (TANK) - TANK-binding kinase

1(TBK1)/ inhibitor of nuclear factor  $\kappa$ B kinase subunit  $\epsilon$  (IKK $\epsilon$ ).<sup>4</sup> In the present study, we set out to determine whether HCoV-HKU1 S and M proteins homologous to their counterparts in SARS-CoV might have similar properties. We also mapped the functional domains in SARS-CoV S and M proteins that are required for their respective UPR-activating and IFN-antagonising activity.

## Methods

This study was conducted from January 2011 to December 2012. HCoV-HKU1 may be cultured in primary human ciliated airway epithelial cells.<sup>5</sup> Nonetheless, success was limited and the virus remains unculturable in most laboratories. Lack of an infectious clone of HCoV-HKU1 further hampers functional analysis. At this stage, mechanistic studies could only be performed through analysis of cloned HCoV-HKU1 S and M genes as well as their mutants. Therefore, all experiments were carried out with cultured HEK293 or 293FT cells transiently transfected with expression vectors for SARS-CoV and HCoV-HKU1 S and M proteins or their truncated mutants.

## Results

### UPR activation by SARS-CoV and HCoV-HKU1 S proteins

We have reported the activation of binding immunoglobulin protein (Grp78) and heat shock

protein 90kDa beta member 1 (Grp94) promoters by SARS-CoV S protein.<sup>2</sup> Grp78 and Grp94 are robust UPR markers. They function as molecular chaperones and are swiftly induced in the UPR. We found that the transcriptional activity driven by the Grp94 promoter was stimulated to a similar extent by S proteins of both SARS-CoV and HCoV-HKU1 (Fig 1). HCoV-HKU1 S protein activated the Grp94 promoter in a dose-dependent manner and with equal potency when compared with SARS-CoV S protein. For another comparison,  $\beta$ -galactosidase was also overexpressed. The activity of Grp94 promoter was minimally or very mildly affected by this large foreign protein. Similar results were obtained with the Grp78 promoter and to a lesser extent with the DNA damage-inducible transcript 3 (C/EBP homologous protein; CHOP) promoter. Activation of Grp78 and Grp94 promoters by S proteins required eukaryotic translation initiation factor 2-alpha kinase 3 (PERK) kinase. Thus, S proteins from both coronaviruses exhibited similar activity to induce ER stress and to activate UPR signalling.

### Definition of UPR-activating domain in S protein of SARS-CoV

SARS-CoV S protein is proteolytically processed into S1 and S2 subunits. To determine whether

the S1 (amino acids 1-770) or S2 (amino acids 771-1255) subunit is required for UPR activation by SARS-CoV S protein, we compared them side by side for activation of Grp78 and Grp94 promoters. The activity of Grp78 and Grp94 promoters was not affected by S2, but induced fully by S1. To compare with HCoV-HKU1 S protein, the cleavage of which inside cells remains uncertain, similar regions matching the S1 and S2 subunits of SARS-CoV S protein were interrogated for their ability to activate Grp78 and Grp94 promoters. Neither S1 nor S2 of HCoV-HKU1 was capable of activating these promoters. The UPR-activating domain in SARS-CoV S1 subunit was further dissected to the central region (amino acids 201-400).

### Suppression of type I IFN production by M protein of SARS-CoV, but not HCoV-HKU1

We have demonstrated that SARS-CoV M protein is capable of antagonising type I IFN production.<sup>3</sup> To assess whether HCoV-HKU1 M protein behaves similarly, we compared the two M proteins for their ability to induce IFN- $\beta$ . We used mitochondrial antiviral-signalling protein (MAVS), a mitochondrial adaptor protein that transmits the activation signal, to boost IFN production. The steady-state level of IFN- $\beta$  transcript in transfected HEK293 cells

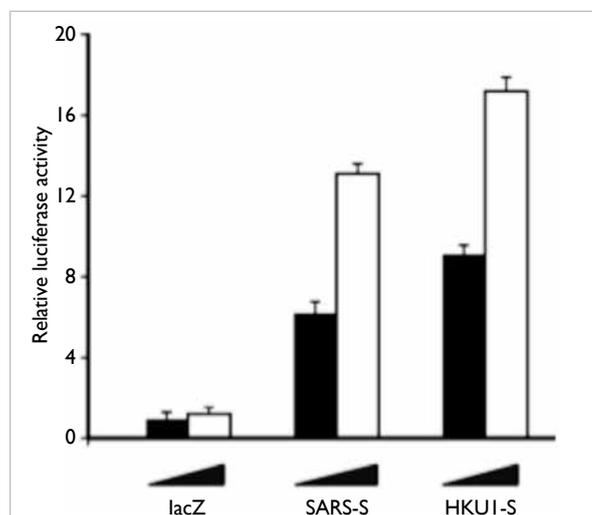


FIG 1. Activation of UPR signalling by S proteins: 293FT cells were transfected with the indicated pGrp94-Luc luciferase reporter and expression constructs. Cells were harvested 36 hours after transfection and dual luciferase assay was performed. Amounts of expression plasmids for lacZ and S proteins were progressively increased. Relative luciferase activity was derived from readouts of firefly luciferase activity normalised to readouts of *Renilla* luciferase activity. Activity recovered from cells receiving lacZ vector alone was set as 1. Results represent means from triplicate experiments with error bars indicating the SD (Reproduced with permission from the Cell and Bioscience and the Cellular and Molecular Immunology).

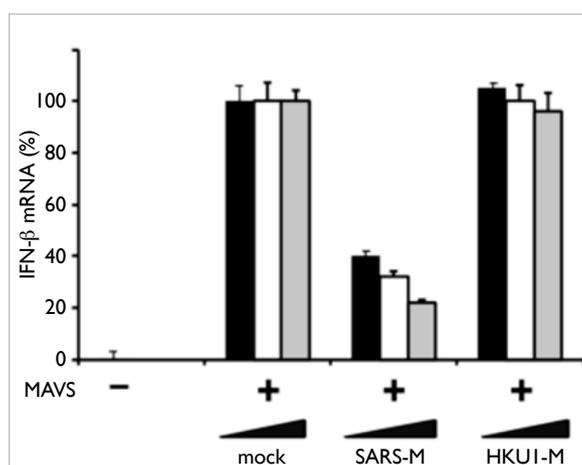


FIG 2. Inhibition of IFN- $\beta$  production by M protein of SARS-CoV (SARS-M) but not of HCoV-HKU1 (HKUI-M): HEK293 cells were transfected with the indicated plasmids and harvested at 30 hours after transfection. Real-time RT-PCR was carried out to quantify the levels of IFN- $\beta$  and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts. A fixed amount of MAVS expression vector and progressively escalating amounts of SARS-M and HKUI-M expression plasmids were used. Relative expression levels of IFN- $\beta$  mRNA were derived from  $2^{-C_t(\text{GAPDH}) - C_t(\text{IFN-}\beta)}$  and normalised to levels from cells mock-transfected with empty expression vector, which were set as 100% (Reproduced with permission from the Cell and Bioscience and the Cellular and Molecular Immunology).

expressing or not expressing SARS-CoV and HCoV-HKU1 M proteins was then monitored by real-time RT-PCR. Interestingly, M protein of SARS-CoV but not of HCoV-HKU1 mitigated the IFN-inducing activity of MAVS (Fig 2). Similar results were also obtained with RIG-I and TBK1. Additional luciferase reporter assays lent further support to the notion that suppression of IFN production by M protein is specific to SARS-CoV and not seen in HCoV-HKU1.

### IFN antagonism is mediated through TM1 of SARS-CoV M protein

Three transmembrane domains (TM1: 1-38 amino acids; TM2: 51-69 residues; and TM3: 76-85 residues) followed by a cytoplasmic endodomain (86-221 amino acids) are found in SARS-CoV M protein. To map the IFN-antagonising domain in SARS-CoV M protein, a series of truncated mutants designated M1, M2, TM1', TM2' and TM3' were constructed and tested in a stepwise approach. First, the three TMs in M1 were indispensable for the suppression of IFN production by SARS-CoV M protein. Second, TM1 of the three TMs was absolutely required for IFN antagonism. Among the three mutants named TM1', TM2' and TM3', which respectively contain TM1 (amino acids 1-38), TM2 (amino acids 51-69) and TM3 (amino acids 76-85) domains fused to the endodomain for the purpose of maintaining protein stability, only TM1' was able to suppress IFN- $\beta$  promoter activity activated by RIG-I, MAVS or TBK1. Neither TM2' nor TM3' showed any IFN-antagonising activity. Since TM2, TM3 and endodomain were not required for IFN antagonism, only TM1 was essential and probably sufficient for the suppressive effect on IFN production.

### Discussion

We compared the UPR-activating and IFN-antagonising activities of S and M proteins of SARS-CoV and HCoV-HKU1. The two S proteins displayed a similar profile of UPR-activating properties with the ability to activate Grp78, Grp94 and CHOP promoters but not UPR element enhancer. The two S proteins also possessed a distinct UPR-activating domain. The S1 subunit of SARS-CoV sufficed to activate the UPR, but no UPR-modulating activity was seen in its counterpart in HCoV-HKU1. Although SARS-CoV and HCoV-HKU1 S proteins had distinct UPR-activating domains, they exerted similar modulatory effects on UPR signalling.

Our findings on the activation of UPR by SARS-CoV and HCoV-HKU1 S proteins have important implications for therapeutic intervention. Pharmaceutical UPR modulators have been developed and extensively tested for diseases including viral infection. Inhibition of PERK

kinase has a negative impact on cytomegalovirus replication, but a small-molecule UPR activator also displays broad-spectrum antiviral activity. In this regard, our analysis of the activation of UPR by S proteins might lay the foundation for further assessment of the utility of UPR modulators for the treatment of SARS and HCoV-HKU1 infection.

SARS-CoV M protein does not share its IFN-antagonising property with HCoV-HKU1 M protein. Its IFN antagonism is mediated by the TM1 domain (amino acids 1-38), which targets it to the Golgi apparatus where it associates with TRAF3 to impede the interaction with TBK1 and IKK $\epsilon$ . Our findings provide additional molecular details for suppression of IFN production by SARS-CoV M protein. Our definition of a small TM1 domain that mediates immune evasion will pave the way for rational design and development of new immunosuppressive agents. In this regard, both peptide mimetics and recombinant proteins that mimic the action of TM1 might prove useful.

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