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# Roles of spike protein in the pathogenesis of SARS coronavirus

## Key Messages

1. Infection with SARS coronavirus (SARS-CoV) induces a cellular stress condition known as the unfolded protein response (UPR). UPR induction is mediated primarily by viral spike (S) protein. The modulation of UPR by S protein involves activation of PERK protein kinase. Other branches of the UPR pathways controlled by IRE1 and ATF6 proteins, respectively, are not involved.
2. The protease inhibitor Ben-HCl effectively suppresses SARS-CoV infection by blocking virus entry. Viral infectivity is associated with the cleavage of S protein by the cellular protease factor Xa.
3. Two new aspects of the interaction between SARS-CoV S protein and the cell have been defined. These have important implications in the pathogenesis of SARS, providing opportunities for developing vaccines and antivirals against SARS-CoV.
4. Counteracting the UPR and targeting the cleavage of S protein with small molecule pharmaceutical agents represent two new anti-SARS-CoV strategies.
5. The receptor-binding domain of S protein delivered via adeno-associated virus can efficiently induce mucosal immunity and provide long-term protection against SARS-CoV infection.

## Introduction

Severe acute respiratory syndrome (SARS) is a potentially fatal infectious disease caused by the SARS coronavirus (SARS-CoV). Like other coronaviruses, SARS-CoV is an enveloped and positive-stranded RNA virus that has a large genome of ~30 kb. It replicates in the cytoplasm and its life cycle is closely associated with the endoplasmic reticulum (ER). The viral activities have a profound impact on ER function. In particular, SARS-CoV hijacks the ER to process its structural and non-structural proteins.<sup>1</sup>

In eukaryotes, the ER is the processing factory for proteins destined for secretion or membrane insertion.<sup>2</sup> Accumulated nascent and unfolded SARS-CoV proteins in the ER lumen during replication can rapidly exceed its folding capacity, thereby perturbing its normal cellular function.

Perturbation of ER function causes stress. Stress of ER activates multiple cell signalling pathways to regulate gene expression at both transcriptional and translational levels. These pathways, collectively termed the unfolded protein response (UPR), adjust the biosynthetic burden and capacity of the ER to maintain homeostasis. To date, three key proximal sensors of UPR, namely ATF6, IRE1 and PERK, have been identified.<sup>2</sup> Unfolded protein response can have both beneficial and detrimental effects during viral infection. To survive ER stress, viruses have developed different strategies to modulate UPR for their own benefits<sup>3</sup> but it is not known if, and in what ways, coronaviruses affect UPR in infected cells.

SARS-CoV S protein is a multifunctional protein that plays pivotal roles in the biology and pathogenesis of SARS-CoV. It has been shown that S protein mediates viral infection by binding to cellular receptor ACE2 and thus inducing membrane fusion. The other functional regions of S protein have not yet been defined. Specifically, it is not understood whether proteolytic cleavage of S affects viral infectivity. Our previous data suggest that the region close to the putative cleavage site might be influential in viral infection.<sup>4</sup>

The aim of this project was to shed light on the molecular and cellular basis of SARS-CoV pathogenesis.

## Methods

This study was conducted from February 2005 to January 2007. The S gene and other viral genes of the SARS-CoV were subcloned and expressed in cultured mammalian cells. Pseudotyped SARS-CoV/HIV (pseudovirus) bearing S protein of SARS-CoV was also constructed. Properties of S protein were characterised in S-gene-transfected, SARS-CoV-infected and pseudovirus-infected cells using Western blotting, luciferase reporter assay, confocal immunofluorescence microscopy and immunoprecipitation. Additionally, recombinant S protein was also obtained and biochemically analysed in vitro.

## Results

### *Infection with SARS-CoV induces ER stress*

To investigate whether infection with SARS-CoV might have an impact on ER stress, we used commercial antibodies for GRP78/94 to determine whether their

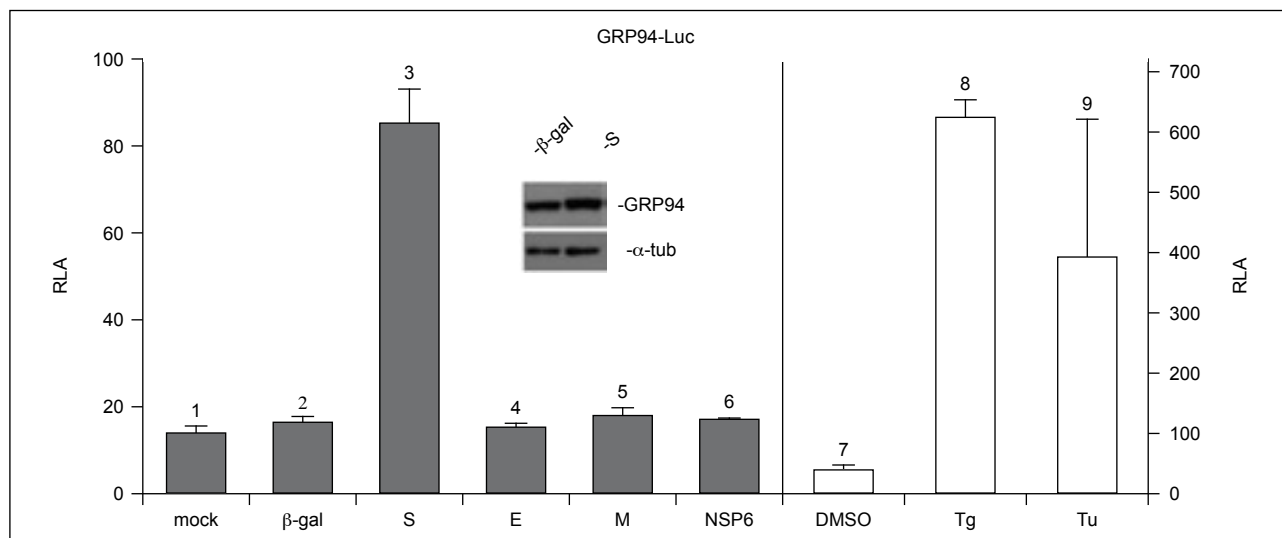
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**Fig 1. Influence of SARS-CoV proteins on UPR: SARS-CoV S protein activates GRP94 promoter (reproduced with permission from the American Society for Microbiology)**

293FT cells were transfected with pGRP94-Luc plus an expression vector for the indicated protein. Control cells transfected with pGRP94-Luc alone were treated with DMSO, Tg (300 nM) or Tu (5 µg/ml) for 16 hrs. Cells were harvested 48 hrs post transfection for dual luciferase assay. Expression levels of GRP94 and  $\alpha$ -tubulin ( $\alpha$ -tub) in  $\beta$ -galactosidase ( $\beta$ -gal)- and S-expressing cells were verified by Western blotting (inset)

expression is induced in SARS-CoV-infected FRhK4 cells. We detected a 4.8-fold increase in the steady-state level of GRP94 in SARS-CoV-infected cells.

To further analyse the influence of SARS-CoV infection on transcriptional activation of GRP94 and GRP78 genes, we transfected luciferase reporter constructs driven by GRP94/78 promoters into Vero cells before infection with SARS-CoV. Our results indicated that infection with SARS-CoV induces ER stress through transcriptional activation of GRP78/94.

#### ***ER stress is induced by SARS-CoV S protein***

To investigate whether different SARS-CoV membrane proteins might perturb the function of ER leading to UPR, we expressed SARS-CoV S, E, M and NSP6 proteins in 293FT cells. We observed that of the four, only S activated transcription from GRP94/78 promoters to ~5-fold (Fig 1, column 3 compared with columns 1 and 2). In the same experiment, treatment with thapsigargin (Tg) and tunicamycin (Tu), two well-known stimuli of ER stress,<sup>2</sup> led to 10~30-fold activation of luciferase expression (Fig 1, columns 8 and 9 compared with column 7). In contrast, none of the other three proteins significantly stimulated GRP94/78 promoters. The activation of GRP94 expression was also confirmed by Western blotting, which showed a 2.4-fold increase of GRP94 protein level in S-expressing cells as normalised to the level of  $\alpha$ -tubulin (Fig 1, inset). Hence, activation of UPR by SARS-CoV is mediated at least partly through S protein.

#### ***Differential regulation of UPR pathways by SARS-CoV S protein***

Stress of ER induces three major pathways of UPR

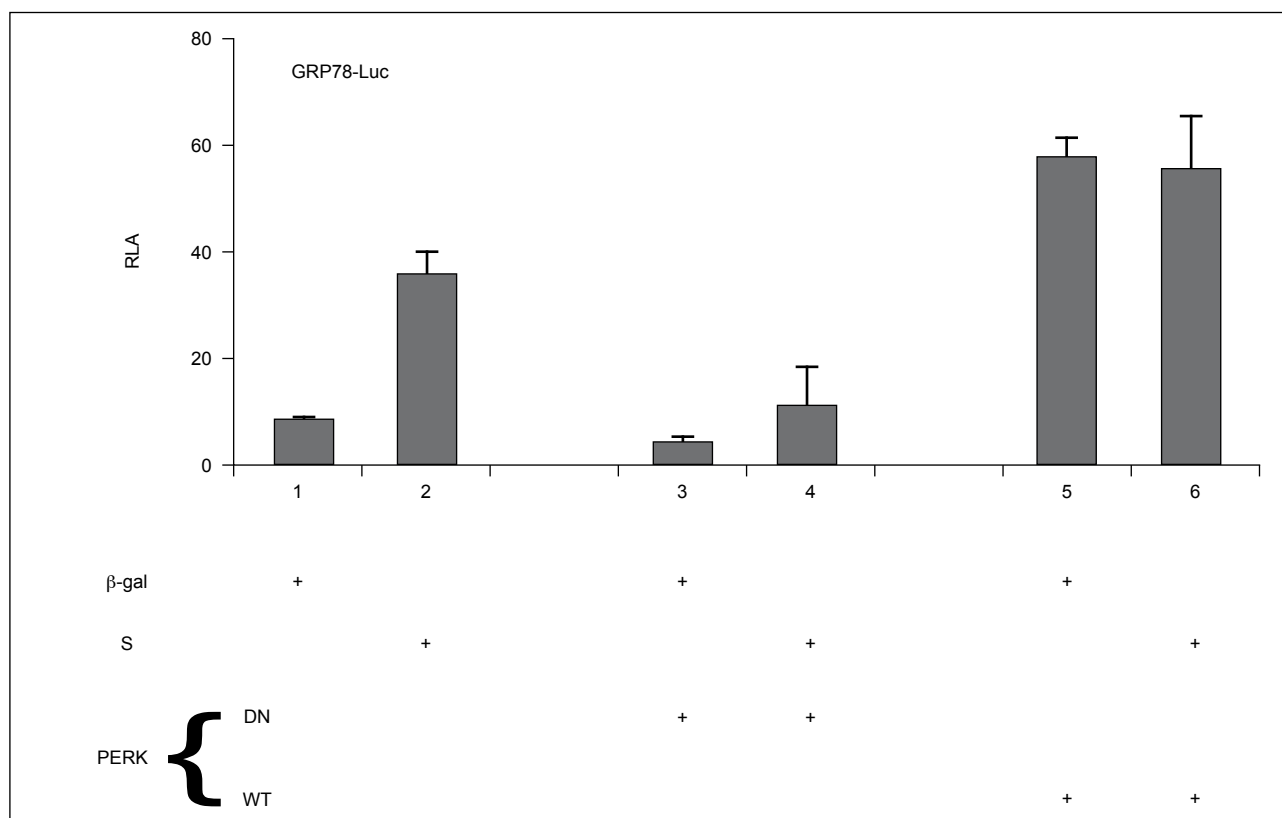
signalling that are mediated through PERK, IRE1 and ATF6, respectively.<sup>2</sup> GRP94/78 promoters have been shown to be upregulated in response to PERK activation and eIF2 $\alpha$  phosphorylation.<sup>4</sup> To investigate whether the activation of GRP94/78 promoters by S protein might be mediated through PERK and eIF2 $\alpha$ , we employed PERK, eIF2 $\alpha$  and their dominant negative (DN) or dominant active (DA) mutants. Interestingly, PERK DN and eIF2 $\alpha$  DN effectively blocked basal and S protein-induced activation of GRP94/78 promoters (Fig 2, columns 3 and 4 compared with columns 1 and 2), whereas PERK wild-type and eIF2 $\alpha$  DA stimulated these promoters (Fig 2, columns 5 and 6 compared with columns 1 and 2). Thus, PERK activity and eIF2 $\alpha$  phosphorylation are required for the activation of ER stress by S protein.

#### ***Protease inhibitor Ben-HCl efficiently suppresses SARS-CoV infection***

To determine if proteolytic cleavage of S protein affects viral infectivity, we screened protease inhibitors for suppressive effects on SARS-CoV infection. Among 13 inhibitors tested, only Ben-HCl had inhibitory activity. In addition, experiments with pseudovirus suggest that Ben-HCl inhibits viral entry into target cells.

#### ***Cleavage of SARS-CoV S protein by factor Xa and its inhibition by Ben-HCl***

Since Ben-HCl is an inhibitor of a panel of proteases, we tested three proteases in this panel, including factor Xa, thrombin and trypsin, for their activities to cleave full-length recombinant S protein of SARS-CoV. Only factor Xa was able to effectively cleave SARS-CoV S protein into S1 and S2 subunits. This cleavage was effectively inhibited by 20 mM Ben-HCl. Similar results were obtained with S protein



**Fig 2. Activation of GRP78 by SARS-CoV S protein requires PERK (reproduced with permission from the American Society for Microbiology)**

293FT cells were co-transfected with pGRP78-Luc and expression vectors for the indicated combinations of proteins. Cells were harvested for dual luciferase assay as in Figure 1

in pseudotyped SARS-CoV/HIV. Thus, both recombinant and pseudoviral S protein can be cleaved by factor Xa.

#### ***S protein was cleaved when the pseudovirus was incubated with the target cells***

To determine if the infectivity of the SARS-CoV/HIV pseudovirus is indeed associated with the cleavage of the S protein by proteases on the target cell membrane, we tested the cleavage of S protein in the culture supernatant by Western blotting, and the infectivity of the pseudovirus in cell lysate using a luciferase assay. Our results indicated that the infectivity of the pseudovirus increased with time and correlated with the size of the cleavage products. The expression of factor Xa in 293T/ACE2 cells was further confirmed by RT-PCR and Western blotting.

#### ***Intranasal vaccination of recombinant AAV encoding RBD of S potently induces mucosal immune responses and provides long-term protection against SARS-CoV infection***

Systemic, mucosal, and cellular immune responses and long-term protective immunity induced by RBD-AAV were characterised in a BALB/c mouse model, with comparison of the intramuscular and intranasal routes of administration. Our findings suggest that RBD-AAV can be further developed into a candidate vaccine against SARS. Intranasal vaccination may be the preferred route

of administration due to its ability to induce SARS-CoV-specific systemic and mucosal immune responses and its better safety profile.

#### **Discussion**

Our demonstration of the modulation of ER stress and UPR by SARS-CoV S protein suggests a new role for the S protein after viral entry. This modulation of UPR probably represents a viral strategy to combat the cellular response and to facilitate viral replication. On the other hand, induction of ER stress by S protein has a significant impact on cell homeostasis and may contribute to viral pathogenesis. For example, UPR is activated in response to the release of ER calcium and it will be of interest to see whether SARS-CoV might induce sufficient calcium release from ER and cause diarrhoea by acting as an NSP4-like viral enterotoxin.<sup>5</sup>

Modulation of ER stress and UPR by the SARS-CoV reveals a novel opportunity for pharmaceutical intervention in SARS. Due to the importance of ER stress in various human diseases including viral infection, small molecules that specifically counteract ER stress have been under intense investigations.<sup>2</sup> In this regard, one selective inhibitor of eIF2 $\alpha$  dephosphorylation has recently been found to be effective for the inhibition of herpes simplex virus replication.<sup>6</sup> Additionally, drugs that modulate ER stress

have also been shown to inhibit the production of infectious CMV virions.<sup>7</sup> As we are yet to identify effective antivirals for the treatment of SARS,<sup>1</sup> further investigation of the use of various ER stress-modulating pharmaceutical agents for anti-SARS-CoV therapy is warranted.

Our findings that SARS-CoV S protein can be cleaved by factor Xa into S1 and S2 subunits both in vitro and in mammalian cells suggests a plausible mechanism by which SARS-CoV cleaves S protein to facilitate viral infection. As inhibition of this cleavage, using agents such as Ben-HCl, can effectively block viral entry, our work has provided a new target for the development of anti-SARS agents. Additionally, the region surrounding the cleavage site should also be included in candidate vaccines against SARS-CoV.

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*Biochem Biophys Res Commun* 2007;359:174-9. (3) Du L, Zhao G, Lin Y, et al. Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection. *J Immunol* 2008;180:948-56. (4) Du L, Zhao G, Lin Y, et al. Priming with rAAV encoding RBD of SARS-CoV S protein and boosting with RBD-specific peptides for T cell epitopes elevated humoral and cellular immune responses against SARS-CoV infection. *Vaccine* 2008;26:1644-51.

### References

1. Weiss SR, Navas-Martin S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiol Mol Biol Rev* 2005;69:635-64.
2. Schröder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem* 2005;74:739-89.
3. He B. Viruses, endoplasmic reticulum stress, and interferon responses. *Cell Death Differ* 2006;13:393-403.
4. Zheng BJ, Guan Y, Hez ML, et al. Synthetic peptides outside the spike protein heptad repeat regions as potent inhibitors of SARS-associated coronavirus. *Antivir Ther* 2005;10:393-403.
5. Ball JM, Tian P, Zeng CQ, Morris AP, Estes MK. Age-dependent diarrhea induced by a rotaviral non-structural glycoprotein. *Science* 1996;272:101-4.
6. Boyce M, Bryant KF, Jousse C, et al. A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science* 2005;307:935-9.
7. Isler JA, Maguire TG, Alwine JC. Production of infectious human cytomegalovirus virions is inhibited by drugs that disrupt calcium homeostasis in the endoplasmic reticulum. *J Virol* 2005;79:15388-97.