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Roles of Spike Protein in the Pathogenesis of SARS Coronavirus

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Summary

Background – SARS coronavirus (SARS-CoV) spike (S) protein is a large and multifunctional protein that plays pivotal roles in the pathogenesis of viral infection. In particular, S is thought to mediate receptor binding and to induce membrane fusion.

Objective – To take a molecular approach to characterizing the properties of SARS-CoV S protein in cultured mammalian cells, thereby shedding light on its roles in the pathogenesis of SARS.

Methods – S gene and other viral genes of 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 characterized in S-gene-transfected, SARS-CoV-infected and pseudovirus-infected cells using Western blotting, luciferase reporter assays and confocal immunofluorescence microscopy. Additionally, recombinant S protein was also obtained and analysed.

Results – We demonstrate that infection with SARS-CoV induces the UPR in cultured cells. A comparison with M, E and NSP6 proteins indicates that SARS-CoV spike (S) protein sufficiently induces transcriptional activation of several UPR effectors including GRP78, GRP94 and C/EBP homologous protein (CHOP). A substantial amount of S protein accumulates in the ER. The expression of S protein exerts different effects on the three major signaling pathways of the UPR. Particularly, it induces GRP78/94 through PKR-like ER kinase (PERK), but has no influence on activating transcription factor (ATF) 6 or X box-binding protein (XBP) 1. On the other hand, we also provided biochemical evidence to support that SARS-CoV S protein is cleaved into S1 and S2 subunits by cellular proteases such as factor Xa during the course of infection.

Conclusions – First, SARS-CoV S protein specifically modulates the UPR to facilitate viral replication. Second, SARS-CoV S protein is likely cleaved in infected cells.

Implications – Our findings have important implications in the pathogenesis of SARS and in the development of antivirals and vaccines against SARS-CoV.

Introduction

Severe acute respiratory syndrome (SARS) is a highly lethal infectious disease in human caused by a newly recognized coronavirus termed SARS coronavirus (SARS-CoV),¹ close relatives of which have recently been found in various species of bats.² Similar to 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. Particularly, SARS-CoV hijacks the ER to process its structural and nonstructural proteins.³

In eukaryotes, the ER is the processing factory for proteins destined for secretion or membrane insertion.⁴ During the replication of SARS-CoV, substantial amounts of viral proteins are produced. Some of them such as the spike (S) and matrix (M) proteins are heavily modified transmembrane proteins.³ This raises the possibility that the accumulation of nascent and unfolded SARS-CoV proteins in the lumen of ER might rapidly exceed its folding capacity, thereby perturbing the normal cellular function of ER.

Perturbation of ER function causes stress. ER stress activates multiple cell signaling pathways to regulate gene expression at both transcriptional and translational levels. These pathways collectively termed unfolded protein response (UPR) adjust the biosynthetic burden and capacity of the ER to maintain homeostasis.⁴ To date, three key proximal sensors of the UPR, namely activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and PKR-like ER kinase (PERK), have been identified. All three are ER-resident transmembrane proteins and they govern three branches of the UPR signaling that activate different subsets of genes encoding ER chaperones and folding enzymes required for protein folding, maturation and degradation.⁴

The effects of the UPR could either be beneficial or detrimental to viral infection. For instance, protein chaperones produced in response to ER stress enhance the folding of viral proteins, but elevated expression of protein degradation factors could lead to inactivation of these proteins. To survive ER stress, viruses have developed different strategies to modulate the UPR. For one example, infection with hepatitis C virus (HCV) has dual effects on the UPR: it activates the promoter of glucose-regulated protein 78 (GRP78) and GRP94 through PERK and ATF6 to stimulate the folding of viral proteins, but suppresses the IRE1-X box-binding protein 1 (XBP1) pathway of the UPR, which governs protein degradation, is suppressed by HCV.⁵ Likewise, human cytomegalovirus (CMV) activates PERK, IRE1 and XBP1, but suppresses ATF6 and a protein degradation factor. Therefore, different viruses differentially regulate the UPR for their own benefits.⁶ However, whether and how coronaviruses impact the UPR in infected cells are not understood.

SARS-CoV S protein is a multifunctional protein that plays pivotal roles in the biology and pathogenesis of SARS-CoV. S has been demonstrated to mediate viral infection, by binding through

the receptor-binding domain (RBD) to cellular receptor angiotensin-converting enzyme 2 (ACE2),⁷ and subsequently inducing membrane fusion through heptad repeats 1 and 2. However, the other functional regions or sites in the S protein important for viral infection have not yet been defined.

In most other members of the coronavirus family, the S protein is cleaved into S1 (receptor binding domain) and S2 (membrane fusion domain).³ A conformational change accompanied with this cleavage triggers membrane fusion. As a new member of the coronavirus family, SARS-CoV shares with other coronaviruses some similarity and common features in the amino acids of the S protein. It has been found that the S protein of SARS-CoV is also cleaved into S1 and S2 domains, but the impact of cleavage on viral infectivity remains unclear.⁸ In this regard, we have shown that SARS-CoV infectivity was inhibited over 99.99% in cell cultures by two peptides corresponding to the sequences proximal to the potential S1 and S2 cleavage site, suggesting that the region might be influential in viral infection.⁹

The aim of this project is to shed light on the molecular and cellular basis of SARS-CoV pathogenesis. Our work was conducted as originally proposed. All aims in the approved application were addressed fully. Particularly, we have studied the oligomerization of S, characterized the receptor-binding domain in S, defined the cleavage of S by cellular protease and its significance to infectivity, and documented the activation of ER stress and UPR by S.

The study of SARS-CoV S is an area of intense investigation and we are in a tight competition with other investigators around the world. New progress on other aspects of SARS-CoV S protein has been reported every 2 or 3 months. Some questions concerning the cellular receptor (original aim d2.3), oligomerization of S (original aim d1.2), and membrane fusion (original aim d3) have also been fully addressed by other groups during the course of our research.¹⁰ Our results in connection to these questions have largely been reported by others in the literature. Some of these results are therefore not worthy of further investigation or publication. Taking this into consideration, we focused on two major issues as proposed in our original application and obtained significant results. We are now taking a lead in this area of research internationally.

The first part of the project focuses on influence of SARS-CoV on ER stress and the UPR. This is an extension to aims d1 and d4 of our original proposal, which involve biochemical and biological characterization of S as well as the study on the influence of S on ER stress. For the first time, we demonstrated the general effect of SARS-CoV infection on ER stress and the modulation of the UPR by SARS-CoV S protein. Our findings pertinent to this issue (aims d1 and d4) have been published as one full article in *Journal of Virology* (publication 1).

The second part of our study concerns the characterization of receptor-binding domain (RBD) in S and the biological significance of the cleavage of S protein to viral infectivity. This is an extension to aims d2 and d3 of our proposal, which involve characterization of receptor binding and fusogenic

activities of S. The RBD of S was studied in detail and the importance of this domain in eliciting protective immunity in vaccine development was established. In addition, we found that a protease inhibitor inhibits SARS-CoV infection and blocks viral entry into cultured cells. We also demonstrated that both recombinant and pseudoviral S protein is effectively cleaved into S1 and S2 by Factor Xa. Importantly, this cleavage was found to be associated with viral infectivity. It is noteworthy that proteolytic cleavage is thought to be the physiological trigger of membrane fusion mediated by S. Particularly, our infectivity assay used in the study is an indirect measurement of fusogenic activity mentioned in our original proposal. Our findings derived from this part of the study (aims d2 and d3) have been published as a short paper in *Biochemical and Biophysical Research Communications*, a full article in *Journal of Immunology*, and another full article in *Vaccine* (publications 2-4).

Methods

Virus and cells – The GZ50 strain of SARS-CoV was propagated in FRhK-4 or Vero cells in a Biosafety Level 3 laboratory as described.¹¹ For reporter assays, Vero cells were transfected with GRP94/78-Luc 24 hours before infection with SARS-CoV. For inactivation of SARS-CoV, virus was exposed to UVC irradiation (254 nm) for 15 min.¹² To assay for the inhibition of SARS-CoV infection, protease inhibitors including Ben-HCl were tested using plaque reduction assay.¹¹

Plasmids – Expression plasmids for eIF2 α mutants were kindly provided by Dr. David Ron. Human PERK and mutant were a generous gift from Dr. Ronald Wek. pCHOP-Luc reporter plasmid was from Dr. Nai Sum Wong. pGRP78-Luc and pGRP94-Luc constructs were supplied by Dr. Kazutoshi Mori. Both promoters have multiple copies of ER stress response element (ERSE). cDNAs encoding SARS-CoV S, E, M and NSP6 proteins were PCR-amplified from molecular clones of SARS-CoV subgenome. Sequences of primers used are available upon request.

Antibodies – Rabbit polyclonal anti-V5, mouse monoclonal anti- α -tubulin and anti- β -actin were from Sigma. Rat monoclonal anti-GRP94 and mouse monoclonal anti-calnexin were from Affinity Bioreagents. Rabbit polyclonal antiserum against SARS-CoV S protein was from Imgenex. Rabbit polyclonal antiserum against total eIF2 α was from Santa-Cruz. Rabbit polyclonal antiserum against eIF2 α phosphorylated on serine 51 was from Cell Signaling. Monoclonal anti-Xa was from R&D Systems. Monoclonal antibodies against S1 (S1-121B8) and S2 (S2-102D7 or -119F6) have been described.¹³

Luciferase reporter assays – Dual luciferase reporter assays were performed using a reagent kit from Promega.¹⁴ Luminescence was measured with an LB9570 luminometer (EG&G). Relative luciferase activity (RLA) was calculated by normalizing readouts of firefly luciferase to those of *Renilla* luciferase.

Western blotting – Western blot analysis was carried out as described.¹⁴ Briefly, cells were lysed in RIPA buffer supplemented with 2 mM phenyl methyl sulfonyl fluoride and other protease inhibitors (Roche). Equal amounts of protein were separated by SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore). Blots were blocked with 5% nonfat milk diluted in Tris-buffered saline (pH 7.6) containing 0.5% Tween 20 (TBS-T), followed by incubation with primary antibodies. After TBS-T washes, blots were further incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham). Proteins were visualized using chemiluminescence detection kits from Amersham.

Confocal immunofluorescence microscopy – Confocal microscopy was performed as previously described.^{14,14} Briefly, transfected cells were fixed in ice-cold acetone/ methanol (1:1) for 10 min. Cells were washed with phosphate-buffered saline (PBS, pH7.4) and blocked with 3% BSA in PBS. The cells were then incubated with the appropriate antibodies. The cover-slips containing cells were mounted on a glass slide using Mowiol prepared in glycerol-PBS. Confocal immunofluorescence microscopy was then performed on BioRad MRC1024 system and the images were captured with the help of the LaserSharp software.

Construction and test of pseudotyped SARS-CoV/HIV – Pseudotyped SARS-CoV/HIV (pseudovirus) bearing full-length S protein of SARS-CoV was as described.¹⁵ The pseudovirus was quantified by measuring p24 level using a Vironostika MicroELISA kit (Biomerieux bv Boxtel, Netherlands). To assess inhibitory effect of Ben-HCl on the infection of pseudovirus, 293T cells expressing ACE2 (293T/ACE2) were seeded into 96-well tissue culture plates at 10^4 cells/well one day before infection. The cells were pre-incubated with Ben-HCl at different concentrations for 30 min at 37°C, followed by infection with varying concentrations of pseudovirus. The infectivity of the pseudovirus was determined by measuring relative luciferase activities.

Cleavage of S protein – The cleavage by Factor Xa was tested with full-length recombinant S protein (Protein Sciences Corporation). In brief, S protein (1.55 µg) was incubated with Factor Xa (0.5 U) in the presence or absence of 20 mM Ben-HCl (Novagen) for 16 h at room temperature. The cleavage assay was also performed with a pseudovirus containing 3 ng of p24.

Detection of Factor Xa transcription by RT-PCR – Transcription of Factor Xa in 293T/ACE2 cells was tested by RT-PCR. Total cellular RNA was extracted using RNeasy Mini kit (Qiagen) and cDNA was synthesized using random primers and SuperScript II RT kit (Invitrogen). PCR reaction was performed with 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72 °C for 1 min, followed by a final extension at 72°C for 10 min.

Immunoprecipitation – Detection of Factor Xa expression in 293T/ACE2 cells at the protein level was performed by immunoprecipitation followed by Western blotting. β-actin was included as a house-keeping protein control. Immunoprecipitation was done using EZview Red Protein A Affinity Gel kit in accordance with the manufacturer's instructions (Sigma). Precipitated proteins (30 µg) were separated on SDS-PAGE and analyzed by Western blotting as described above.

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 GRP94 and GRP78 to determine whether their expression is induced in SARS-CoV-infected FRhK4 cells. As such, we detected a 4.8-fold increase in the steady-state level of GRP94 in SARS-CoV-infected cells. This finding is generally consistent with results from global proteomic analysis of SARS-CoV-infected cells.

To further analyze 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. We then harvested cells at 12-48 hours post infection (hpi) for measurement of relative luciferase activity (RLA). Progressively increased RLA values in SARS-CoV-infected cells indicated the activation of GRP94/78 promoters. The RLA values remained constant in cells incubated with SARS-CoV inactivated by ultraviolet irradiation. Thus, 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-encoded transmembrane proteins might perturb the function of ER leading to the UPR, we expressed SARS-CoV S, E, M and NSP6 proteins in 293FT cells. We observed that among the four, only S activated transcription from GRP94/78 promoters to ~5-fold (Figure 1, column 3 compared to columns 1 and 2). In the same experiment, treatment with thapsigargin (Tg) and tunicamycin (Tu), two well-known stimuli of ER stress,⁴ led to 10~30-fold activation of luciferase expression (Figure 1, columns 8 and 9 compared to 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 shows a 2.4-fold increase of GRP94 protein level in S-expressing cells as normalized to the level of α -tubulin (Figure 1, inset). In addition, S could modestly stimulate transcription from the promoter of CHOP, a major component of the ER stress-induced apoptosis pathway, whereas it did not significantly affect translation of ATF4. The expression of S and other SARS-CoV proteins (E, M and NSP6) was verified by Western blotting and confocal immunofluorescence microscopy. Hence, our results suggest that differential activation of the UPR by SARS-CoV is mediated at least in part through S protein.

Differential regulation of the UPR pathways by SARS-CoV S protein. ER stress induces three major pathways of the UPR signaling that are mediated through PERK kinase, IRE1 and ATF6,

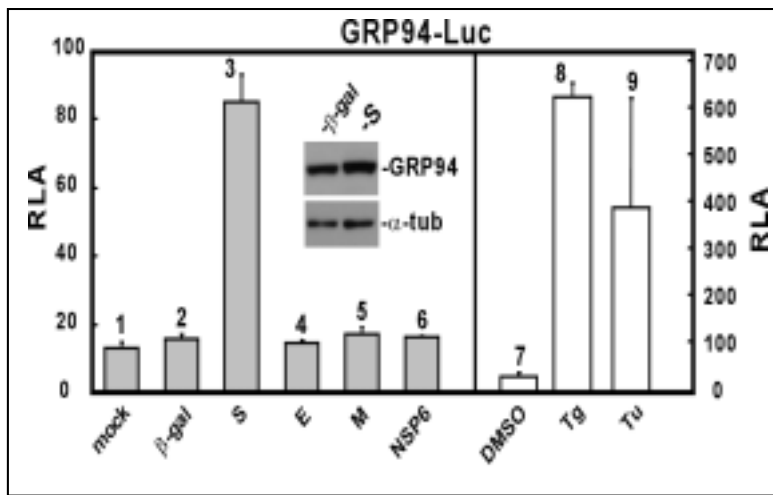


Figure 1. Influence of SARS-CoV proteins on the UPR: SARS-CoV S protein activates GRP94 promoter. 293FT cells were transiently co-transfected with pGRP94-Luc plus a pLenti-based expression vector for the indicated protein. Control cells transfected with pGRP94-Luc alone were treated with DMSO, Tg (300 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 α-tubulin (α-tub) in β-galactosidase (β-gal)- and S-expressing cells were verified by Western blotting (inset).

respectively.⁴ GRP94/78 promoters have been shown to be upregulated in response to PERK activation and eIF2α phosphorylation.⁴ To investigate whether the activation of GRP94/78 promoters by SARS-CoV S protein might be mediated through PERK and eIF2α phosphorylation, we employed expression plasmids for PERK, eIF2α and their dominant negative (DN) or dominant active (DA) mutants (Figure 2). We observed that PERK DN and eIF2α DN effectively blocked basal and S protein-induced activation of GRP94/78 promoters (Figure 2, columns 3 and 4 compared to columns 1 and 2), whereas PERK wild-type and eIF2α DA stimulated these promoters (Figure 2, columns 5 and 6 compared to columns 1 and 2). Thus, PERK activity and eIF2α phosphorylation are required for the activation of ER stress by S protein. In further support of this, infection with SARS-CoV or expression of S alone led to an about 4-fold elevation of the relative amount of phosphorylated eIF2α.

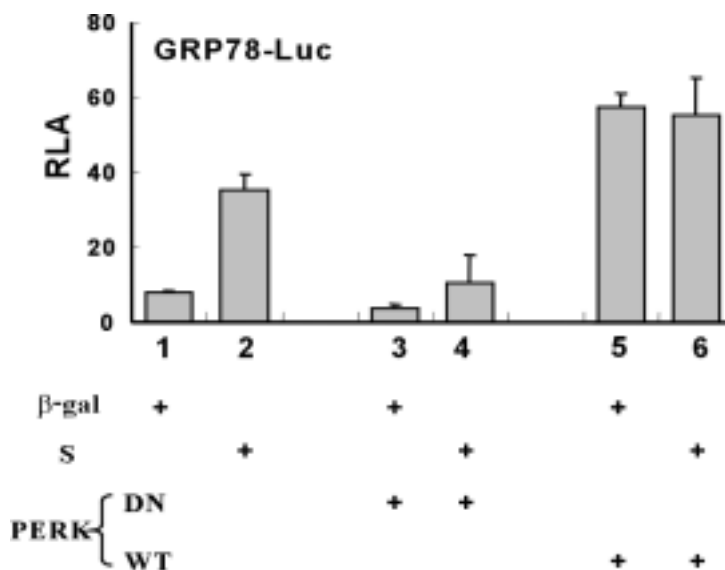


Figure 2. Activation of GRP78 by SARS-CoV S protein requires PERK. 293FT cells were co-transfected with pGRP78-Luc and expression vectors for the indicated combinations of proteins. Cells were harvested for dual luciferase assay as in Figure 1.

We also assessed the influence of SARS-CoV infection and of S protein on the other two pathways of UPR signalling. We found that SARS-CoV S protein specifically activated PERK, but did not significantly affect IRE1/XBP1 or ATF6. Interestingly, infection with SARS-CoV probably induces IRE1 and XBP1 activation at a low level through an unknown but S-independent mechanism.

Protease inhibitor Ben-HCl efficiently suppresses SARS-CoV infection. To ask 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 has inhibitory activity. To confirm that the inhibition occurs at the level of viral entry, the inhibitory effect of Ben-HCl was evaluated using a pseudotyped SARS-CoV/HIV virus expressing S protein of SARS-CoV. We observed that readings of relative luciferase activity decreased progressively with increased concentrations of Ben-HCl. Treatment with 20 mM Ben-HCl resulted in nearly complete inhibition of pseudovirus infection. The results further demonstrated that Ben-HCl could indeed inhibit 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 3 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. Moreover, Factor Xa could also cleave S protein in pseudotyped SARS-CoV/HIV. When the pseudovirus was incubated with Factor Xa at room temperature for 2 h, pseudoviral S protein was partially cleaved by Factor Xa into S1 and S2, and the cleavage increased with the concentration of Factor Xa. When the incubation time was extended to 16 h, pseudoviral S protein was almost completely cleaved by 0.5 U of Factor Xa. Notably, the cleavage of pseudoviral S protein by Factor Xa could be inhibited by 80 mM Ben-HCl. Thus, both recombinant and pseudoviral S protein could be cleaved by Factor Xa.

S protein is 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 by luciferase assay after the pseudovirus (3 ng) was incubated with 293T/ACE2 cells for 2 and 4 h in the presence or absence of Ben-HCl (20 mM). Extra bands were evident in the lane with pseudovirus-infected culture supernatant without Ben-HCl. These bands reacted with anti-S1 (121B8) and anti-S2 (119F6)

antibodies, respectively, indicating that the cleavage increased with time. In contrast, no such bands were detected in the pseudovirus infected supernatant in the presence of Ben-HCl. Luciferase assay further showed that the infectivity of the pseudovirus increased with time and correlated with the amounts of 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 characterized in a BALB/c mouse model, with comparison of the intramuscular (i.m.) and intranasal (i.n.) routes of administration. Our results demonstrated that: 1) the i.n. vaccination induced a systemic humoral immune response of comparable strength and shorter duration than the i.m. vaccination, but the local humoral immune response was much stronger; 2) the i.n. vaccination elicited stronger systemic and local specific cytotoxic T cell responses than the i.m. vaccination, as evidenced by higher prevalence of IL-2 and/or IFN- γ -producing CD3⁺/CD8⁺ T cells in both lungs and spleen; 3) the i.n. vaccination induced similar protection as the i.m. vaccination against SARS-CoV challenge in mice; 4) higher titers of mucosal IgA and serum-neutralizing Ab were associated with lower viral load and less pulmonary pathological damage, while no Ab-mediated disease enhancement effect was observed; and 5) the vaccination could provide long-term protection against SARS-CoV infection. Taken together, our findings suggest that RBD-AAV can be further developed into a candidate vaccine for prevention of SARS and that i.n. 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

SARS-CoV is highly pathogenic and its S protein is thought to play a pivotal role in viral pathogenesis. In particular, S protein mediates receptor binding, induces membrane fusion, and elicits neutralizing immune response.³ Our demonstration of the modulation of ER stress and the UPR by SARS-CoV S protein suggests a new role for S after viral entry. This modulation of the UPR likely represents a viral strategy to combat cellular response and to facilitate viral replication. The effect of S on the UPR was shown mainly in the transcriptional activation of intraluminal ER chaperones GRP94/78 through PERK and eIF2 α phosphorylation. Increased expression of these chaperones would enhance the folding and processing of SARS-CoV proteins that are abundantly expressed during viral replication. The induction of CHOP by S is milder. In addition, S had little or no influence on ATF4, XBP1 or ATF6. Because CHOP is a major regulator of ER stress-associated apoptosis,³ activation of CHOP at an early stage of viral infection is undesirable. Hence, SARS-CoV benefits from selective modulation of these events by S protein.

Viral modulation of ER stress and the UPR signaling has just begun to be understood. Other viruses such as HCV and CMV modulate ER stress and the UPR by activating PERK and inhibiting XBP1 or EDEM.^{5,16} It is natural that different viruses adopt different strategies in their modulation of the UPR.⁶ We showed that SARS-CoV S protein used a unique strategy to deal with ER stress by stimulating PERK but exerting no effect on IRE1 or ATF6. Consistent with this, infection with SARS-CoV does not induce apoptosis at least in some cells or at an early stage of infection.³ It remains to be elucidated whether S protein from other coronaviruses might also exhibit similar modulatory activity on ER stress.

Induction of ER stress by S protein has a significant impact on cell homeostasis and may contribute to viral pathogenesis. For example, the UPR is activated in response to the release of ER calcium as induced by drugs such as Tg.⁴ On the other hand, the disruption of calcium homeostasis is a feature of viral enterotoxin exemplified by rotavirus NSP4.¹⁷ Because SARS-CoV also replicates commonly in small and large intestines leading to diarrhea,³ it will be of particularly great interest to see whether SARS-CoV proteins might sufficiently induce calcium release from ER and cause diarrhea by acting as an NSP4-like viral enterotoxin. In another perspective, a recent study has revealed a link between ER stress and systemic inflammatory response.¹⁸ This induction of acute phase response (APR) genes by ER stress is mediated by regulated cleavage of CREB-H transcription factor and probably other transmembrane bZIP factors such as LZIP/CREB3 in the same protein subfamily.¹⁹ These consequences of ER stress and the UPR could be relevant to the pathogenesis of SARS.

Modulation of ER stress and the UPR by SARS-CoV reveals a novel opportunity for pharmaceutical intervention of 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.⁴ In this regard, one selective inhibitor of eIF2 α dephosphorylation has recently been found to be effective for the inhibition of herpes simplex virus replication.²⁰ Additionally, drugs that modulate ER stress have also been shown to inhibit the production of infectious CMV virions.¹⁶ Because antivirals highly effective for the treatment of SARS have not been identified,³ further investigations on the use of various ER stress-modulating pharmaceutical agents for anti-SARS-CoV therapy are warranted.

SARS-CoV infection involves the binding of RBD in the S1 domain with ACE2 on the surface of the host cell. The conformational changes triggered by the binding between RBD and ACE2 result in the interaction of HR1 and HR2 to form a coiled-coil structure in the S2 domain, which then facilitates membrane fusion.⁷ Our findings acquired in this project indicated that SARS-CoV S protein could be cleaved by Factor Xa into S1 and S2 subunits both *in vitro* and in mammalian cells.

We further explored if the cleavage of S protein indeed plays a critical role in SARS-CoV infection. Our results demonstrated that: (1) Factor Xa can effectively cleave the S protein in the SARS-CoV/HIV pseudovirus into S1 and S2 subunits and this cleavage is inhibited by Ben-HCl ; (2) S protein is cleaved into S1 and S2 subunits when the pseudovirus was incubated with target cells (293T/ACE2) and the level of the cleavage product correlates with viral infectivity; and (3) the target cells express Factor Xa, a membrane-bound protease. Collectively, our study suggested a plausible mechanism by which SARS-CoV cleaves the S protein to facilitate viral infection.

The infection of SARS-CoV not only involves the binding of RBD with its receptor ACE2 and the fusion between viral envelope and host cell membrane, but also is associated with the cleavage of the S protein by proteases on the cell membrane, such as Factor Xa. It has been reported that the S protein of SARS-CoV forms trimeric peplomer on the surface of the virion.^{9,21} The cleavage of the S protein may trigger virus-cell fusion subsequent to the binding with ACE2 receptor and the dissociation of trimeric S protein to monomers.

Development of effective vaccines and antiviral drugs against SARS remains necessary. Previous studies have explored several targets for developing vaccines and antivirals, including RBD and HR2.^{9,15} In addition, small molecules targeting SARS-CoV enzymes, such as 3CLpro protease, papain-like protease 2, and helicase, can also inhibit viral replication.¹¹ Because inhibition of the cleavage of S protein into functional S1 and S2 subunits using agents such as Ben-HCl can effectively block viral entry, our work has provided a new target for development of anti-SARS agents. Additionally, the region surrounding the cleavage site should also be included in candidate vaccines against SARS-CoV.

Conclusions

- 1) We demonstrated the induction of ER stress and the UPR by SARS-CoV infection through S protein. Particularly, we showed that S protein modulated ER stress differentially by stimulating PERK but sparing the other two branches of the UPR signaling mediated through IRE1 and ATF6.
- 2) We found that Ben-HCl, a protease inhibitor, is able to effectively suppress SARS-CoV infection by blocking virus entry. In addition, viral infectivity is associated with the cleavage of S protein by Factor Xa.
- 3) We documented the utility of RBD-AAV in inducing mucosal immunity and providing long-term protection against SARS-CoV infection.

Implications

Our work has defined two new aspects of the interaction between SARS-CoV S protein and the cell. Our findings have important implications in the pathogenesis of SARS and have revealed new opportunities for developing vaccines and antivirals against SARS-CoV. Two new anti-SARS-CoV strategies that emerge from our study in this project are counteracting ER stress and targeting cleavage of S protein. We have also provided a new type of candidate vaccine against SARS-CoV based on RBD-AAV.

Dissemination

We have published our findings in mainstream international journals. One paper appeared in *Journal of Virology*, one of the best journals in the field of virology. Another paper was published in *Journal of Immunology*, another mainstream international biomedical journal. We have also presented our results in conferences including the International Symposium of the Society of Chinese Bioscientists in America. We will continue to do so with our unpublished data.

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Appendices

Full version of four papers published on this project:

- 1) *Journal of Virology*, **80**(18):9279-9287, 2006
- 2) *Biochemical and Biophysical Research Communications*, **359**(1):174-179, 2007
- 3) *Journal of Immunology*, **180**(2):948-956, 2008
- 4) *Vaccine* **26**(13):1644-1651, 2008