

A study on antigenicity and receptor-binding ability of fragment 450–650 of the spike protein of SARS coronavirus

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Abstract

The spike (S) protein of SARS coronavirus (SARS-CoV) is responsible for viral binding with ACE2 molecules. Its receptor-binding motif (S-RBM) is located between residues 424 and 494, which folds into 2 anti-parallel β -sheets, β 5 and β 6. We have previously demonstrated that fragment 450–650 of the S protein (S450–650) is predominantly recognized by convalescent sera of SARS patients. The N-terminal 60 residues (450–510) of the S450–650 fragment covers the entire β 6 strand of S-RBM. In the present study, we demonstrate that patient sera predominantly recognized 2 linear epitopes outside the β 6 fragment, while the mouse antisera, induced by immunization of BALB/c mice with recombinant S450–650, mainly recognized the β 6 strand-containing region. Unlike patient sera, however, the mouse antisera were unable to inhibit the infectivity of S protein-expressing (SARS-CoV-S) pseudovirus. Fusion protein between green fluorescence protein (GFP) and S450–650 (S450–650-GFP) was able to stain Vero E6 cells and deletion of the β 6 fragment rendered the fusion product (S511–650-GFP) unable to do so. Similarly, recombinant S450–650, but not S511–650, was able to block the infection of Vero E6 cells by the SARS-CoV-S pseudovirus. Co-precipitation experiments confirmed that S450–650 was able to specifically bind with ACE2 molecules in lysate of Vero E6 cells. However, the ability of S450–510, either alone or in fusion with GFP, to bind with ACE2 was significantly poorer compared with S450–650. Our data suggest a possibility that, although the β 6 strand alone is able to bind with ACE2 with relatively high affinity, residues outside the S-RBM could also assist the receptor binding of SARS-CoV-S protein.

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Keywords: SARS-CoV; S protein; Receptor-binding motif; B cell epitope

Introduction

SARS-associated coronavirus (SARS-CoV) is a positive-stranded RNA virus of the *Coronaviridae* family. It is the causative agent of severe acute respiratory syndrome (SARS) and infected over 8000 people around the world in 2002–2003 (Peiris et al., 2003; Rota et al., 2003). The genome of SARS-CoV encodes several structural proteins including the spike (S) glycoprotein, nucleocapsid protein, membrane protein and envelope protein (Marra et al., 2003). The S protein of SARS-CoV is consisted of 1255 amino acid residues with approxi-

mately 25% homology to that of the other human CoVs (Ho et al., 2004; Spiga et al., 2003). It mediates viral binding to angiotensin-converting enzyme-2 (ACE2), a cell surface zinc peptidase (Li et al., 2003). The S protein receptor-binding domain (S-RBD) was mapped to a fragment between residues 318 to 510 (Wong et al., 2004). Li et al. (2005) have recently solved the crystal structure of S-RBD complexed with ACE2 at 2.9 Å resolution. Their results demonstrate that S-RBD contains 2 subdomains: a core (residues 318 to 423) and an extended loop (residues 424 to 494). The latter, referred as the S protein receptor binding motif (S-RBM), is formed by 2 anti-parallel β -sheets (β 5 and β 6) folding into a gently concave surface, which makes all contacts with the ACE2 molecule (Li et al., 2005). Interestingly, 9 out of the 14 amino acid residues of S-RBM in direct contact with ACE2 are in the β 6 strand (residues 450 to

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494), suggesting a possibility that the $\beta 6$ fragment alone might be able to bind with ACE2 with considerable affinity.

As the receptor binding site of SARS-CoV, S-RBD is the most likely target for neutralizing Abs against the virus. Several groups have mapped a number of B cell epitopes in, or around, the S-RBD region (Hu et al., 2005; Hua et al., 2004, 2005; Lu et al., 2005; Wang et al., 2003), although the exact location and conformational structure of such epitopes remain to be clarified. Our previous results demonstrated that SARS patients mounted early and strong humoral responses against fragment 450–650 of the S protein (S450–650) (Zhao et al., 2005a, 2005b), which contains the $\beta 6$, but not $\beta 5$, strand of the S-RBM. We herein analyze the receptor binding ability of S450–650 and also its antigenicity in terms of recognition by Abs.

Results

Expression and purification of recombinant truncated polypeptides of S450–650 and their fusion products with GFP

Preparation of recombinant S450–650 was as previously described (Zhao et al., 2005a, 2005b). DNA encoding truncated fragments covering the sequence of S450–650, namely S-I (450–510), S-II (511–560), S-III (546–595), S-IV (581–630) and S-V (601–650), were amplified by PCR using vector pET28a-S450–650 (encoding for S450–650 of SARS-CoV) as template. The resultant products were inserted into prokaryotic expression vector pET28a. DNA encoding GFP was inserted downstream the S protein genes for preparation of GFP fusion proteins S450–650-GFP, S450–510-GFP and S511–650-GFP (Fig. 1A). Glycine- and serine-rich linkers of various length have been widely used in protein engineering since these linkers may offer little structure hindrance to folding proteins in their own constructs (Liu et al., 2005; Nardella et al., 2004). A linker sequence (GSGGSGS) was therefore introduced between S fragment and GFP. The recombinant products, expressed in *E. coli*, were purified to more than 90% homogeneity using Ni columns and examined by SDS-PAGE electrophoresis (Figs. 1B and 2).

Possible B cell epitopes in S450–650

Previous studies have demonstrated the presence of B cell epitopes in S-RBD by screening synthetic or recombinant polypeptides covering its sequence in ELISA or WB assays (Chen et al., 2004; Zhang et al., 2004). To further map the B cell epitopes in the S450–650 region, a set of convalescent patient sera (PT31, PT41, PT42, PT53, and PT58), all tested positive in SARS-CoV-based ELISA as previously described (Zhao et al., 2005a), were screened in WB experiments against recombinant S-I, S-II, S-III, S-IV and S-V. As illustrated in Fig. 2, S-II (511–560) and S-IV (581–630) were strongly recognized by all 5 patient sera, while none of the other overlapping constructs produced detectable signals. These results imply that fragments between residues 511–545 and 596–600 represent either linear B cell epitopes or parts of conformational B cell epitopes predominantly recognized by human Abs produced following an infection of SARS-CoV.

BALB/c mice were s.c. immunized with recombinant S450–650 emulsified in CFA and subsequently tested for serum Abs against the immunizing Ag and also its truncated polypeptides in WB assays. In contrary to that obtained with patient sera, the mouse anti-serum strongly recognized S-I, weakly recognized S-III and S-IV but did not recognize S-V (Fig. 2).

Comparison of the neutralization ability of patient sera and mouse antiserum

By co-transfecting 293T cells with a SARS-CoV-S-protein-expressing vector and a defective HIV-1-genome-expressing luciferase, we prepared S-protein expressing pseudovirus. The pseudovirus thus obtained was able to infect ACE2-positive Vero E6 cells in vitro, evidenced by the approximately 100,000 folds increase of luciferase activity in the infected cells (Fig. 3A). The system was employed to compare the neutralization ability of patient sera and mouse antiserum. As shown in Fig. 3B, convalescent patient sera (PT31 and PT53), but not mouse anti-S450–650 antiserum, were able to specifically neutralize S-protein-expressing pseudovirus infection of Vero E6 cells, implying that Ab specificity for linear epitopes in the $\beta 6$ strand is not necessarily associated with neutralization ability. Both human and mouse sera tested herein were unable to neutralize the infectivity of VSV-G pseudovirus (Fig. 3C), confirming the specificity of the system.

Staining of Vero E6 cells using S450–650 or its GFP fusion proteins

To assess the receptor binding ability of the S450–650 fragment, Vero E6 cells were treated with recombinant S450–650 followed by either convalescent patient serum (PT31), or control serum sample (HDS), or mouse anti-S450–650 antiserum (MAS), or normal mouse serum (NMS). S-RBD-Fc is a fusion protein between the S-RBD domain (residues 318–510) and Fc fragment of human IgG1. It has been shown to be able to bind with ACE2 receptor with high affinity (Kuba et al., 2005). As controls, S-RBD-Fc and human IgG-Fc were used to treat Vero E6 and also ACE2-negative 293T cells. After washes, the cells were subsequently treated with FITC-conjugated secondary Abs for FACS analysis. As illustrated in Figs. 4A and B, S450–650-treated Vero E6 cells were specifically recognized by patient serum PT31 or mouse anti-S450–650 antiserum, indicating specific binding of the recombinant S450–650 to the cell surface. To further locate the site for receptor binding within the 450–650 construct, we next employed fusion proteins S450–510-GFP, S511–650-GFP and S450–650-GFP for direct staining of Vero E6 and 293T cells. As expected, S450–650-GFP was able to label Vero E6, but not 293T cells and deletion of the 450–510 sequence completely abolished its ability of doing so (Figs. 4D and E). It is noteworthy that the intensity of Vero E6 cell staining by S450–650 (Fig. 4B) and S450–650-GFP (Fig. 4D) was approximately 50% of that by S-RBD-Fc (Fig. 4C), while the fluorescence intensity of the S450–510-GFP-stained cells was only 1/20 of that stained by S-RBD.

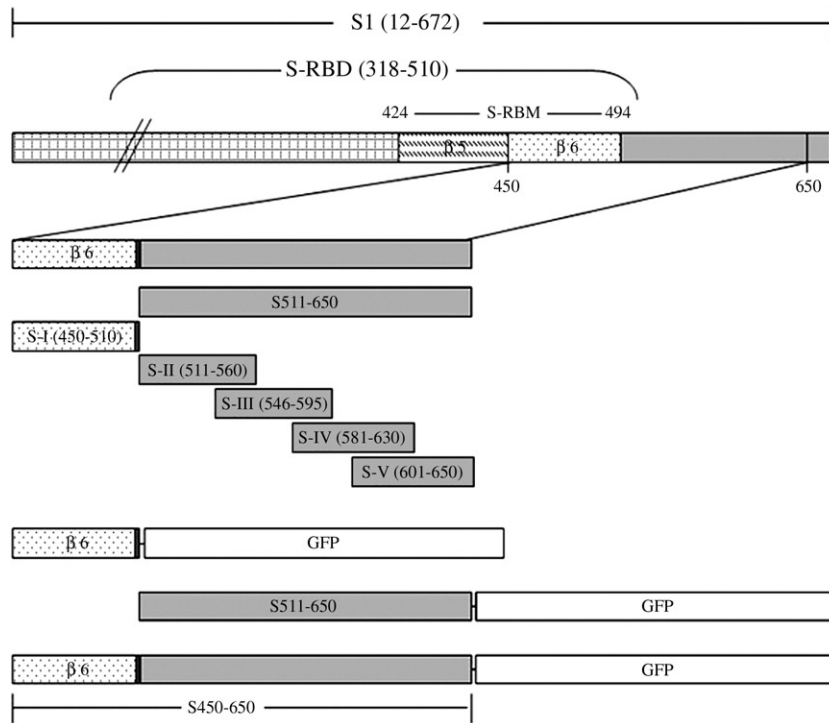
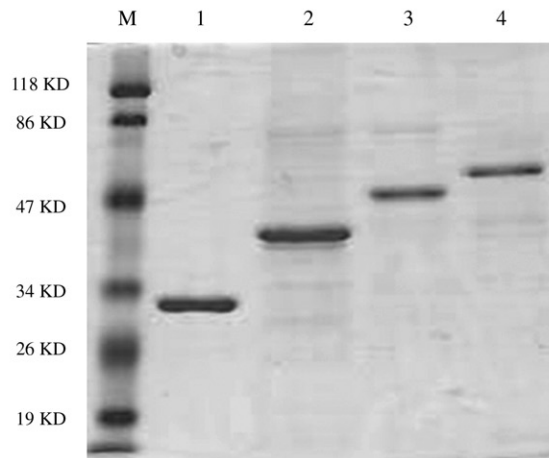
A: Relative location of S-RBD, S-RBM and S450-650**B: SDS-PAGE**

Fig. 1. Relative locations of the S-RBD, S-RBM and recombinant polypeptides prepared in this study. (A) Recombinant S450–650 and truncated polypeptides covering its sequence, including S-I (450–510), S-II (511–560), S-III (546–595), S-IV (581–630), S-V (601–650) and S511–650 were expressed in *E. coli*. Recombinant GFP and its fusion proteins with S protein fragments were also prepared. (B) The purifications product of GFP (lane 1) and its fusion proteins with S-I (S450–510-GFP, lane 2), S511–650 (S511–650-GFP, lane 3) or S450–650 (S450–650-GFP, lane 4) were analyzed by SDS-PAGE electrophoresis and visualized by Commassie blue staining. Molecular weight markers were loaded in the left-hand-side lane.

Recombinant S450–650 blocks the SARS-CoV-S pseudovirus infection of Vero E6 cells

Fig. 5A illustrates that the S-RBD-Fc fusion protein was able to block the pseudovirus infection of Vero E6 cells with an IC_{50} of 0.4 μ M. If the recombinant S450–650 polypeptide can specifically bind with cellular receptors for S protein, it should also be able to block the infection of host cells by SARS-CoV or

SARS-CoV-S pseudovirus. As shown in Fig. 5B, S450–650 inhibited the infection of Vero E6 cells by SARS-CoV-S pseudovirus with an IC_{50} of 0.8 μ M, while the S511–650 polypeptide did not show any significant inhibitory effect. Coinciding with the direct staining results illustrated in Fig. 4, the S-I fragment (residues 450 to 510) was much less efficient in blocking the SARS-S pseudovirus infection of Vero E6 cells (Fig. 5B).

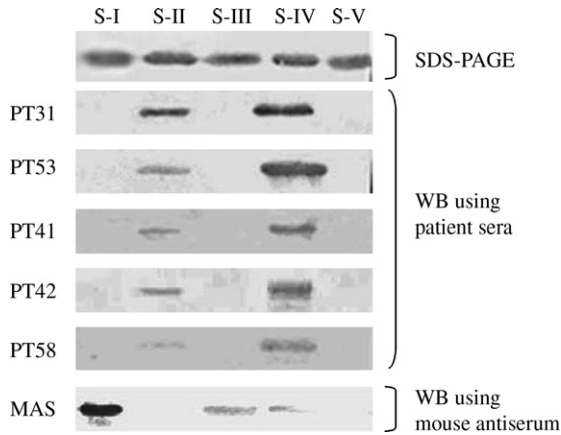


Fig. 2. Possible B cell epitopes in S450–650. Recombinant constructs S-I (450–510), S-II (511–560), S-III (546–595), S-IV (581–630) and S-V (601–650) were run on SDS-PAGE 12% gels (0.5 µg in each lane) and visualized by Commaissie blue staining (top row). The protein bands were transferred onto nitrocellulose membranes for WB with convalescent patient sera (PT31, PT41, PT42, PT53, PT58) or mouse anti-S450–650 antiserum (MAS) as first Abs. HRP-conjugated goat anti-human, or goat-anti-mouse, IgG Abs were employed, respectively, as detection Abs.

Co-precipitation of S450–650 and ACE2 in vitro

In the experiment shown in Fig. 6, Vero E6 cells were treated with recombinant S450–650, washed and then lysed using lysis buffer. The lysate was treated sequentially with PT31 serum and protein A/G-agarose for precipitation of the S450–650/ACE2 complexes. The resultant precipitates were run on SDS-PAGE gels and assayed by WB with anti-ACE2 Abs as first Abs. Fig 6B illustrates that the PT31-precipitated materials contained ACE2 molecules (120 kDa band specifically recognized by goat anti-human ACE2 Abs). In parallel experiments, the S450–650-treated cell lysate was precipitated using goat anti-human ACE2 polyclonal Abs and then the precipitates analyzed by WB using PT31 serum as the first Abs. A 31 kDa protein band, corresponding to the size of the S450–650 polypeptide, was clearly visualized (Fig. 6C).

Discussion

Identification of neutralizing epitopes in the S protein is of great interest for vaccine design and also our understanding of anti-SARS-CoV humoral immunity in patients. It has been demonstrated that the S-RBD (residues 318–510) contains immunodominant B cell epitopes and some of which are recognized by neutralizing Abs (He et al., 2005; He et al., 2004; Sui et al., 2004). Presumably, the neutralizing epitopes are located within the S-RBM region (residues 424–494) which forms direct contacts with ACE2 (Li et al., 2005). However, this has so far not been confirmed by experimental data. In our WB assays, the β6 strand (i.e. SI or S450–510) was not recognized by convalescent patient sera (Fig. 2). Although the β6 strand was predominantly recognized by antisera from mice after immunization with the S450–650 construct (Fig. 2), such Abs did not show neutralization ability against SARS-CoV-S pseudovirus infection (Fig. 3). By immunizing mice with the S-RBD-Fc, which adopts a natural fold of the S-RBD (Kuba et al., 2005), He et al. (2005) generated a panel of mAbs, two of which were specific for linear epitopes between residues 450 and 510, neither of these mAbs was able to neutralize viral infection. This is in agreement with our data, shown in Fig. 3, and indicates that the lack of neutralization ability of the mouse Abs specific for linear sequences in the β6 strand is likely a genuine phenomenon rather than the result of improper presentation in the context of the peptides.

It should also be emphasized, however, that the above results do not exclude the possibility that the β6 strand, or the whole S-RBM fold formed by β5 and β6 strands together, contains conformation- dependent B cell epitopes predominantly recognized by neutralizing Abs. In WB assays, the reducing and denaturing nature of SDS-PAGE would only allow protein Ags to be recognized in linear form.

Our WB results also show that 2 fragments near the S-RBM (residues 511 to 545 and 596 to 600) were specifically recognized by all 5 patient sera tested (Fig. 2). Whether binding of human Abs to these epitopes contributes to neutralization is still unclear,

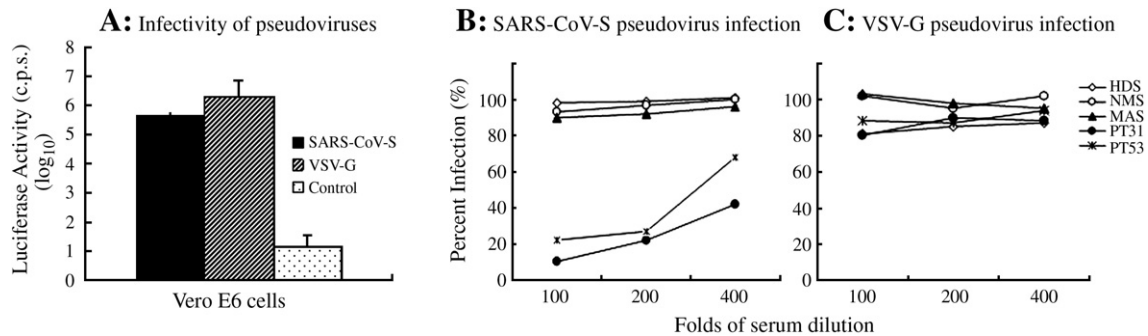


Fig. 3. Comparison for neutralization ability of patient sera and mouse antiserum. (A) Infectivity of the SARS-CoV-S pseudovirus. Preparations of SARS-CoV-S pseudovirus, or VSV-G pseudovirus, or mock transfection supernatant (Control) were added to triplicate wells on 96-well plates containing monolayer of Vero E6 cells (8 × 10³ cells/well) and incubated at 37 °C for 1 h. The incubation continued for 48 h after fresh medium replacement and the cells were then lysed and luciferase activity in the lysate determined. The results are expressed as luciferase activity (log₁₀). (B and C) Neutralization assay. Serially diluted serum samples from convalescent patients (PT31 and PT53), or healthy blood donors (HDS), or S450–650-immunized mice (MAS), or naïve BALB/c mice (NMS) were mixed with medium containing SARS-CoV-S, or VSV-G, pseudovirus, and incubated for 30 min at 37 °C. The mixtures were then distributed into triplicate wells in 96-well plates containing monolayer of Vero E6 cells. Luciferase activity in the infected cells was determined 48 h later and the results expressed as percent infection compared with the control group (Vero E6 cells only treated with pseudovirus preparations).

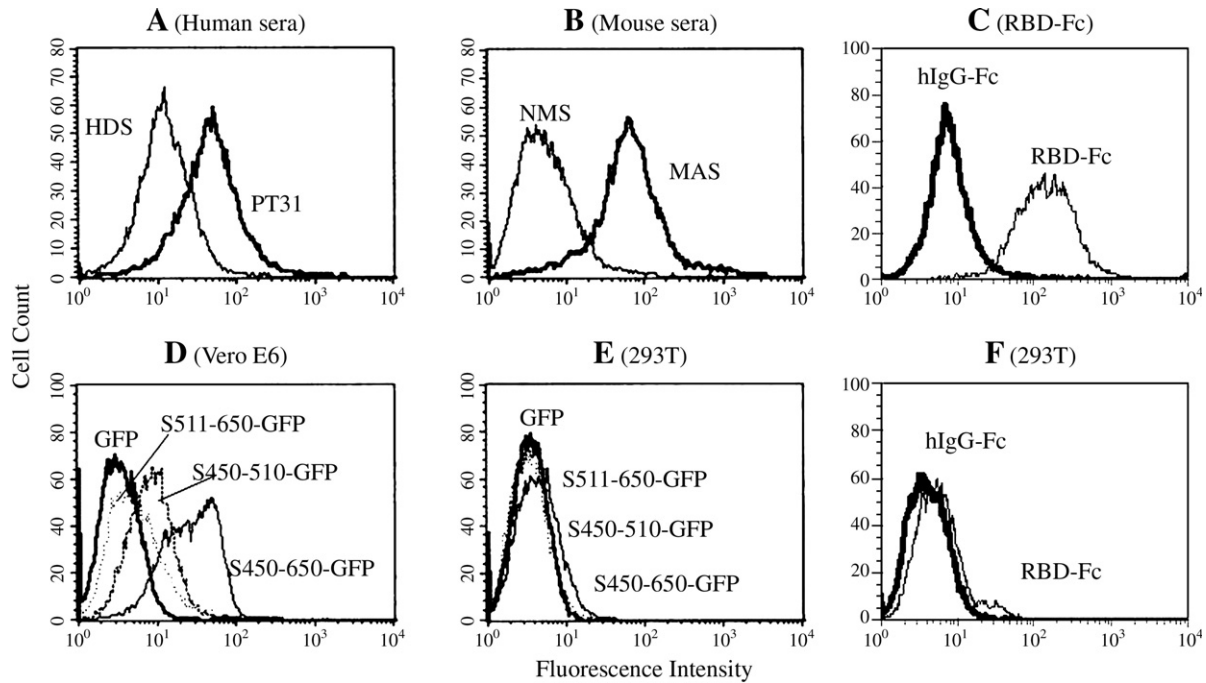


Fig. 4. Staining of Vero E6 cells with S450–650 or its GFP fusion proteins. For indirect staining (A, B), Vero E6 cells were treated with S450–650 (0.5 μM) in PBS at 4 $^{\circ}\text{C}$ for 1 h, followed by convalescent patient serum (PT31), or mouse anti-S450–650 antiserum (MAS), or serum sample from healthy blood donors (HDS), or normal mouse serum (NMS). FITC-conjugated goat anti-human, or goat-anti-mouse, IgG Abs were employed as secondary Abs. As controls, Vero E6 cells and 293T cells were treated with S-RBD-Fc, or human IgG (0.5 μM), at 4 $^{\circ}\text{C}$ for 1 h and followed by FITC-conjugated goat anti-human IgG (C, F). For direct staining, Vero E6 (D), or 293T (E), cells were treated with GFP, S450–510-GFP, S511–650-GFP or S450–650-GFP (0.5 μM) at 4 $^{\circ}\text{C}$ for 30 min. After washes, the cells were subjected to flow cytometric analysis.

although a correlation between S450 and 650 specificity (mostly targeted at the 511–545 and 596–600 epitopes) and neutralization ability of convalescent sera provide circumstantial evidence for this notion (JC Zhao, unpublished observation). Furthermore, data reported by Li et al. (2005) and He et al. (2005) suggest that Ab binding to regions near the S-RBM fold may cause conformational change of the receptor binding sites, resulting in inhibition of the RBD binding to ACE2.

Although the S450–650 construct contains only half of the S-RBM fold ($\beta 6$ but not the $\beta 5$ strand), it is nonetheless able to bind to ACE2 with relatively high affinity, as determined in cellular staining, pseudovirus inhibition assays and co-precipitation experiments (Figs. 4, 5 and 6). This is also supported

by Hu et al. who documented that recombinant S471–503 blocked the binding between S-RBD and ACE2 (Hu et al., 2005). Additionally, our computer modeling results show that the $\beta 6$ strand contributes significantly more than $\beta 5$ in terms of contact area (238 vs. 17 \AA^2) as well as binding energy (139 vs. 30.6 kJ/mol) when interacting with ACE2 (Fig. 7).

An unexpected finding of this study is that construct S450–510, either alone or in fusion with GFP, was relatively poor in binding to ACE2 compared with S450–650, as determined in both Vero E6 cell staining (Fig. 4D) and SARS-CoV-S pseudovirus inhibition (Fig. 5B) experiments. There are several possible explanations for this phenomenon. In the case where GFP-fusion proteins were employed, it is possible that the

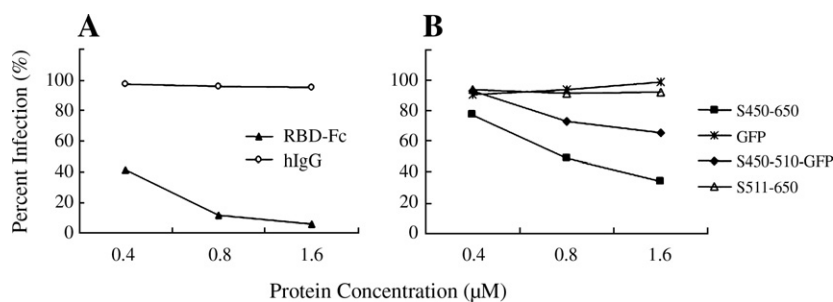


Fig. 5. Pseudovirus infection blocking assay. SARS-CoV-S pseudovirus preparations were mixed with, or without (reference group), RBD-Fc or human IgG and incubated together for 30 min at 37 $^{\circ}\text{C}$. The mixtures were then distributed into triplicate wells containing monolayer of Vero E6 cells seeded on 96-well plates 18 h previously. Luciferase activity in the infected cells was determined 48 h later (A). In a parallel experiment (B), SARS-CoV-S pseudovirus preparations were mixed with, or without (reference group), recombinant GFP, S450–650, S450–510-GFP or S511–650 at 37 $^{\circ}\text{C}$ for 30 min and then used to infect Vero E6 cells. The results are expressed as percent infection (luciferase activity of experimental group compared with that of the reference group).

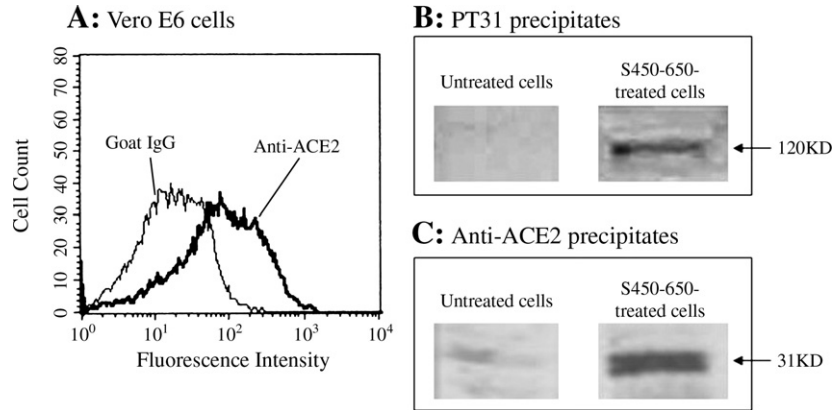


Fig. 6. Co-precipitation of recombinant S450–650 with ACE2 in the lysate of Vero E6 cells. Vero E6 cells (1×10^6) were sequentially treated with goat anti-human ACE2 Abs, or goat IgG (2 μ g), and FITC-conjugated rabbit anti-goat IgG. The stained cells were subjected to flow cytometric analysis for determination of ACE2 expression on the cell surface (A). For the co-precipitation experiment (B), Vero E6 cells (2.5×10^7) were treated with, or without, recombinant S450–650 (15 μ g/ml) at 4 $^{\circ}$ C for 1 h. After washes, the cells were lysed using lysis buffer and the lysate treated with PT31 serum followed by protein-A/G-agarose precipitation. The precipitated materials were run on SDS-PAGE 12% gels and the protein bands transferred to nitrocellulose membranes for WB assays using goat anti-human ACE2 Abs as the first Abs, the detection Abs were HRP-labeled rabbit anti-goat IgG. In a parallel experiment (C), lysate of the S450–650-treated, or untreated, Vero E6 cells were precipitated using goat anti-human ACE2 Abs and protein-A/G-agarose. The precipitates were assayed by WB using PT31 serum (first Ab) and HRP-labeled goat anti-human IgG (detection Ab).

ACE2-binding site on a ~ 5.5 kDa peptide could be physically blocked by the >20 kDa GFP. This blocking effect is significantly lessened in S450–650-GFP construct that binds ACE2 efficiently, perhaps because of a buffering effect of residues 511–650 between $\beta 6$ (i.e. 450–510) and GFP. In addition, by starting the molecule from the middle of the S-RBM fold, the S450–650 might be prone-to-misfold. Thirdly, sequences adjacent to S-RBM may also assist the receptor binding of S-RBM in a yet unknown mechanism. By virtue of the formation of inter or intra-molecular disulfide bonds, the binding of S450–650 polypeptide for ACE2 could be stabilized and enhanced as well. Xiao et al. (2004) found

that a dimeric state of S1 portion of the S protein could contribute to an increased overall affinity that may enhance fusion efficiency. This hypothesis may help explaining the fact that Abs against regions adjacent to the S-RBM exhibit neutralizing potential.

Materials and methods

Molecular biology reagents and cell culture

High fidelity Taq DNA polymerase was purchased from TaKaRa Biotech Co. Ltd. (Shiga, Japan). Restriction enzymes

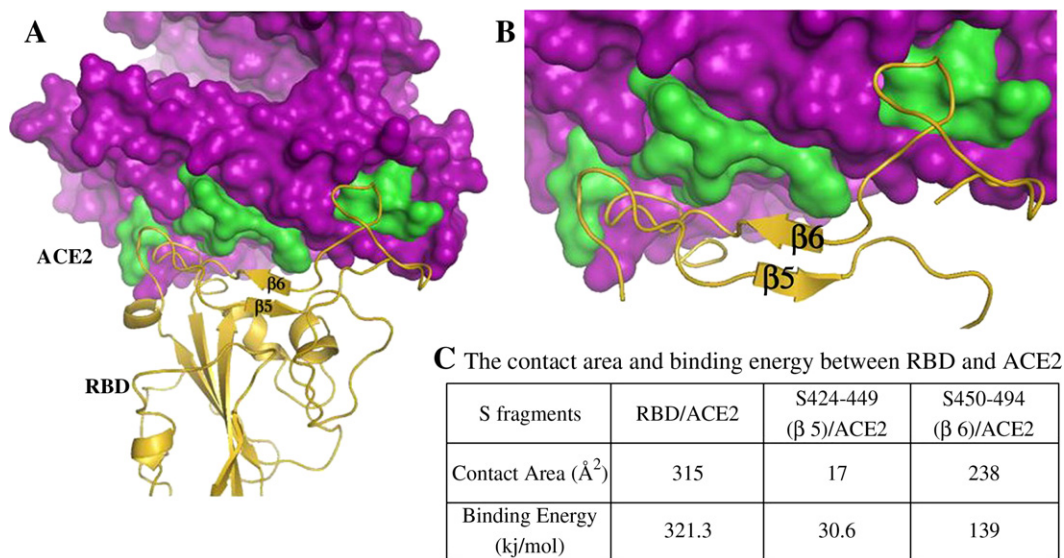


Fig. 7. ACE2 interaction with the $\beta 5$ and $\beta 6$ strands of S-RBM. (A) Crystal structure of the RBD in complex of human receptor ACE2 as reported by Li and co-workers (Li et al., 2005). Details of the binding interface between S-RBM and the ACE2 molecule and calculation results of the contact area and binding energy are shown in panels B and C, respectively. The S protein-binding areas of the ACE2 molecule (purple) are colored in green and the $\beta 5$ and $\beta 6$ strands in gold. The binding energy here is the energy required to disassemble the RBD-ACE2 complex into separate parts. The figure is prepared using PYMOL and contact area and binding energy calculated by SPDBV. PDB file accession number is 2AJF.

and T4 ligase were from Invitrogen (California, USA). A kit for DNA extraction and purification was from Qiagen (Hilden, Germany). *E. coli* strain of BL21 (DE3) was from Stratagene (La Jolla, California, USA). The Ni-nitrilotriacetic acid (Ni-NTA) resin was from Novagen (Darmstadt, Germany). Protein A/G-agarose for immunoprecipitation was supplied by Santa Cruz Biotechnology, Inc. (California, USA). The cell transfection reagent was from Vigorous Biotech Co. (Beijing, China). Luciferase assay reagent was supplied by Promega (Madison, USA). Goat anti-human ACE2 ectodomain polyclonal Abs was purchased from R&D systems (Minneapolis, MN, USA). FITC-conjugated rabbit anti-goat, goat anti-human, goat anti-mouse IgG, horseradish peroxidase (HRP)-labeled goat anti-human and goat anti-mouse IgG were obtained from Zhongshan Biotech Co. (Beijing, China). Vero E6 cells and 293T cells were obtained from ATCC and propagated in DMEM supplemented with 10% fetal bovine serum (Hyclone, Utah, USA).

Subjects and blood samples

Serum samples used in this study were from convalescent SARS patients, collected between July and August 2003 by the Beijing Red Cross Center. The blood samples were processed within 18 h of collection. In addition, a control serum sample (HDS) was prepared by mixing sera from 10 randomly selected healthy blood donors.

Expression and purification of recombinant proteins

Expression vector containing the gene for S450–650 (pET28a-S450–650) was as previously described (Zhao et al., 2005a, 2005b). DNA encoding truncated fragments of S450–650 was PCR amplified using pET28a-S450–650 as template. A vector containing the gene for green fluorescence protein (GFP) was a gift from Dr. L. Barber, Imperial College School of Science, Technology and Medicine, London. For preparation of GFP fusion proteins, a linker sequence (–Gly–Ser–Gly–Gly–Ser–Gly–Ser–) was added between the S protein fragment and GFP using sequence overlapping extension PCR method. The DNA sequences encoding for S protein fragments or their GFP fusion proteins were inserted into pET28a expression vector. Recombinant products were expressed in *E. coli* and purified using Ni-NTA resin following the manufacturer's instructions. The preparation of S-RBD-Fc, a recombinant fusion protein between S-RBD (residues 318–510) and the Fc portion of human IgG1, was expressed in CHO cells and purified by chromatography as described elsewhere (Kuba et al., 2005).

Animal immunization and serum collection

Female BALB/c mice of 6–8 weeks of age, obtained from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China, were immunized s.c. with recombinant S450–650 (50 µg/mouse/100 µl), or PBS, emulsified in complete Freund's adjuvant (CFA, Sigma, USA)

at the base of the tail. Booster immunizations were given on days 15 and 29 with S450–650 emulsified in incomplete Freund's adjuvant (IFA). The mice were bled for sera a week after the third immunization and the serum samples were kept at –80 °C until use.

Immunoprecipitation (IP) and Western blot

The procedure for IP-Western blot assay was according to that by Li et al. (2003). Briefly, Vero E6 cells (2.5×10^7) were treated with, or without, recombinant S450–650 (15 µg) at 4 °C for 1 h. The cells were washed 3 times and then lysed in lysis buffer containing 0.5% NP-40 and protease inhibitors (pepstatin, leupeptin and aprotinin, all from Sigma). After removal of cell debris by centrifugation, the lysate was allowed to mix with either PT31 serum (1:200 diluted) or goat-anti-human ACE2 Abs (2 µg/ml final concentration) at 4 °C overnight. Sera from healthy subjects (HDS) and goat IgG were also included as controls. Protein A/G-agarose was then added and incubated at 4 °C for 1 h, followed by 3 washes. Bound proteins were eluted in Laemmli sample buffer, at 90–100 °C for 10 min, and separated on SDS-PAGE 12% gels. The protein bands were transferred onto nitrocellulose membranes for WB assays using goat anti-human ACE2 Abs or PT31 serum as the first Abs.

The nitrocellulose membrane, on which the recombinant proteins were transferred from SDS-PAGE gels, were blocked at room temperature for 2 h with blocking solution (5% non-fat dry milk) and then incubated for 2 h at room temperature with convalescent sera of SARS patients (1:200 diluted) or antisera from mice after S450–650 immunization (1:1000 diluted). After washes in Tris-buffered saline (TBS, pH7.5) containing 0.05% Tween 20 (TBS-T), the membranes were incubated with HRP-labeled goat anti-human or anti-mouse IgG. 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) was used to visualize the reaction.

Indirect and direct staining of Vero E6 cells

For indirect staining experiments, Vero E6 cells (1×10^6) were treated with recombinant S450–650 (0.5 µM) at 4 °C for 1 h. After 3 washes, the cells were incubated at 4 °C for 30 min in the presence of patient sera (1:100 diluted) or mouse anti-S450–650 antiserum (1/200 diluted). Serum samples from healthy blood donors or naive BALB/c mice were included as controls. Recombinant S-RBD-Fc and human IgG-Fc were also employed to treat Vero E6 cells (0.5 µM, 4 °C for 1 h) as controls. After washes, the cells were incubated with FITC-conjugated goat anti-human or goat-anti-mouse IgG at 4 °C for 30 min and analyzed with FACScan (BD).

For direct staining experiments, Vero E6 cells (1×10^6) were treated with GFP or GFP fusion proteins (0.5 µM) at 4 °C for 30 min, followed by flow cytometric analysis using a FACScan machine. GFP has a single excitation peak at 490 nm and emission peak at 509 nm, which adapts to common fluorescein isothiocyanate (FITC) bandpass of FACS (Gerdes and Kaether, 1996).

Preparation of SARS-CoV-S pseudovirus

Preparation of SARS-CoV-S-protein- expressing pseudovirus was according to the method of Nie et al. (2004). Briefly, 293T cells (5×10^6), seeded in 10 cm diameter tissue culture dishes one day earlier, were co-transfected with defective HIV-1 genome pNL4.3-Luc-R⁻E⁻ (20 µg) and plasmid pVSV-G (10 µg), encoding for vesicular stomatitis virus (VSV) G protein, or plasmid pCMV-S, encoding for the S protein of SARS-CoV, using the Vigorous transfection reagent (Vigorous Biotech Co., Beijing, China). The defective HIV-genome contains the gene for luciferase which can be used as a reporter of successful infection by the pseudovirus. The culture medium was replaced with fresh medium 24 h post-transfection and the cells cultured for additional 24 h. The culture supernatant, containing VSV-G-pseudovirus or SARS-S-pseudovirus, was then harvested and filtered through a 0.45 µm pore-sized filter, followed by centrifugation at 35,000 rpm for 3 h at 4 °C using a Beckman XL-90 ultra centrifuge (Beckman Coulter, Harbor Boulevard, USA). The pseudoviral pellets were resuspended in PBS, titrated, aliquoted and stored at -80 °C until use.

Neutralization and blocking assays using the SARS-CoV-S pseudovirus system

SARS-CoV-S pseudovirus and VSV-G pseudovirus infection of cells and its neutralization were according to Zhang et al. (2004). Briefly, Vero E6 cells were seeded in 96-well plates, at a density of 8×10^3 cells/well, and cultured overnight. Serially diluted serum samples or recombinant proteins were mixed with pseudovirus preparations for 30 min at 37 °C, and then the mixture added to the wells with monolayer of Vero E6 cells. After incubation at 37 °C for 1 h, the mixture in the wells was replaced with fresh medium and the cells were cultured for additional 48 h. After washes, the cells were lysed using the luciferase assay reagent (Promega, UAS) and the luciferase activity in the cell lysate determined using a Veritas microplate luminometer (Turner Biosystems).

Luciferase activity of the reference group (Vero E6 cells treated with pseudovirus alone) was taken as 100% infection. Cells not treated with pseudovirus were included as specificity control and their luciferase activity readings were at least 3 logs lower than that of the reference group in all experiments. Luciferase activity of the experimental groups (Vero E6 cells treated with pseudovirus preparations in the presence of serum Abs or blocking reagents) was compared with that of the reference group and the results, expressed as percent infection, are calculated as follows: $100 \times [\text{Luciferase activity of the experimental groups}] / [\text{Luciferase activity of the reference group}]$.

Statistical analysis

All experiments described here had been repeated at least 3 times. Comparison of the data was performed using the Student's *t* test. Significance was defined as a *P* value < 0.05.

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