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A single amino acid substitution (R441A) in the receptor-binding domain of SARS coronavirus spike protein disrupts the antigenic structure and binding activity

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Abstract

The spike (S) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) has two major functions: interacting with the receptor to mediate virus entry and inducing protective immunity. Coincidently, the receptor-binding domain (RBD, residues 318–510) of SAR-CoV S protein is a major antigenic site to induce neutralizing antibodies. Here, we used RBD-Fc, a fusion protein containing the RBD and human IgG1 Fc, as a model in the studies and found that a single amino acid substitution in the RBD (R441A) could abolish the immunogenicity of RBD to induce neutralizing antibodies in immunized mice and rabbits. With a panel of anti-RBD mAbs as probes, we observed that R441A substitution was able to disrupt the majority of neutralizing epitopes in the RBD, suggesting that this residue is critical for the antigenic structure responsible for inducing protective immune responses. We also demonstrated that the RBD-Fc bearing R441A mutation could not bind to soluble and cell-associated angiotensin-converting enzyme 2 (ACE2), the functional receptor for SARS-CoV and failed to block S protein-mediated pseudovirus entry, indicating that this point mutation also disrupted the receptor-binding motif (RBM) in the RBD. Taken together, these data provide direct evidence to show that a single amino acid residue at key position in the RBD can determine the major function of SARS-CoV S protein and imply for designing SARS vaccines and therapeutics.

Keywords: SARS-CoV; Spike protein; Receptor-binding domain; Epitopes

The spike (S) protein of severe acute respiratory syndrome coronavirus (SARS-CoV), similar to those of other coronaviruses, is a large type I transmembrane glycoprotein, which is incorporated into the viral envelope and provides the virion with a corona-like appearance [1,2]. Unlike those of many other cornaviruses, the S protein of SARS-CoV may not be cleaved in the virus-producing cells [3,4]; however, two domains corresponding to the N-terminal S1 subunit and the C-terminal S2 subunit of processed coronaviruses can be defined by sequence alignment [5,6]. The S1 subunit of coronavirus S protein forms the surface knob-like structure, whereas the S2 subunit is membrane-anchored and forms the stem-like structure beneath the knob [7,8].

The binding of coronavirus to its specific receptor on the target cell is an initial step of infection [7–9]. Angiotensin-converting enzyme 2 (ACE2) is a functional receptor for SARS-CoV [10–12]. The S protein of SARS-CoV can bind to ACE2 with high affinity and mediates viral entry. A 193-residue fragment within S1 domain (residues 318–510) has been defined as a minimal receptor-binding domain (RBD) (Fig. 1) [4,13,14]. Crystal structure of an independently folded RBD bound to human ACE2 reveals that residues 424–494 constitute the receptor-binding motif (RBM) [15]. The S2 domain of SARS-CoV S protein contains a putative fusion peptide and two heptad repeat regions (HR1 and HR2), which can associate to form a six helix bundle comprised of three helices from HR1 that run anti-parallel to three helices from HR2 [16–18].

The second major function of coronavirus S protein is its capacity to elicit neutralizing antibodies and sterilizing

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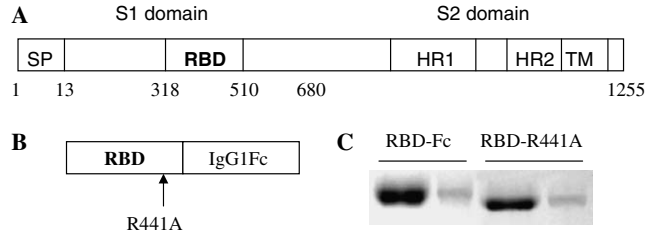

Fig. 1. Schematic diagrams of SARS-CoV S protein and RBD-Fc fusion protein. (A) There is a signal peptide (SP) located at the N-terminus of the S protein. The S1 domain contains a receptor-binding domain (RBD, residues 318–510) and the S2 domain contains two heptad repeat regions (HR1 and HR2) prior to transmembrane domain (TM). (B) Recombinant RBD-Fc molecule consists of RBD and a human IgG1-Fc fragment. R441A was generated by mutagenesis using the QuickChange XL kit. (C) Characterization of purified RBD-Fc and RBD-R441A mutant by SDS–PAGE analysis.

immunity, and is thereby considered as a critical immunogen for vaccine development [19,20]. Similarly, it has been shown that the S protein of SARS-CoV is a major protective antigen among four structural proteins [21]. Several live virus and DNA vaccines expressing the S protein have been tested in preclinical studies [22–24]. Coincidently, the RBD of SARS-CoV S protein is a major target of neutralizing antibodies induced in patients infected with SARS-CoV and in animals immunized with inactivated viruses or S proteins [25–27]. We previously demonstrated that RBD-Fc, a fusion protein containing the RBD linked to Fc portion of human IgG1, is a potent inducer of neutralizing antibodies and has potential to be developed as a subunit vaccine [28,29]. Several conformation-dependent neutralizing epitopes (Conf I–VI) were identified in the RBD [29]. Although the RBD is a major neutralizing domain of SARS-CoV, the S protein also contains neutralizing epitopes in other regions [30–32]. Interestingly, it was recently reported that the major function of full-length S protein, i.e., mediating viral entry and inducing neutralizing antibodies, could be abolished by single amino acid substitutions in the RBD (e.g., R441A) [33]. In this study, we used the RBD-Fc as a model molecule to further investigate the impact of R441A substitution on the immunogenicity and receptor-binding activity of independently folded RBD. Our data are important for understanding the mechanism how a single amino acid residue determines the major function of SARS-CoV S protein.

Materials and methods

Recombinant S proteins and mAbs. The plasmid encoding the receptor-binding domain (residues 318–510) of SARS-CoV S protein Tor2 (Accession No. AY274119), fused with the Fc portion of human IgG1 (RBD-Fc), was previously described [28,29]. RBD-Fc bearing R441A substitution (designated as RBD-R441A) was generated by mutagenesis using the QuickChange XL kit (Stratagene) and verified by DNA sequencing. Each of the recombinant fusion proteins was expressed in 293T cells transfected with the plasmid using Fugene 6 reagents (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol and purified by protein A–Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ). The full-length S protein (FL-S) of SARS-CoV Urbani (Accession No. AY278741) was expressed in expresSF$^{+®}$ insect cells with recombinant baculovirus D3252 by the Protein Sciences Corporation (Bridgeport, CT).

A panel of 26 mAbs specific for the RBD of SARS-CoV S protein was prepared in our laboratory, which include 14 mAbs isolated from mice immunized with RBD-Fc [29], 6 mAbs isolated from mice immunized with FL-S, and 6 mAbs isolated from mice immunized with inactivated SARS-CoV.

Immunization of mice and rabbits. RBD-Fc and its mutant RBD-R441A were, respectively, used to immunize mice and rabbits. Four female BALB/c mice (6 weeks old) per group were subcutaneously immunized with 20 μg of purified proteins re-suspended in PBS plus MLP + TDM Adjuvant (Sigma, Saint Louis, MI) and boosted with 10 μg of the same antigen plus the MLP + TDM adjuvant at 3-week intervals. Four NZW rabbits (12 weeks old) per group were immunized intradermally with 150 μg purified proteins re-suspended in phosphate-buffered solution (PBS, pH 7.2) in the presence of Freund's complete adjuvant (FCA), and boosted three times with freshly prepared emulsion of 150 μg immunogen and Freund's incomplete adjuvant (FIA) at 3-week intervals. Pre-immune sera were collected before starting the immunization and antisera were collected 7 days after each boost. Sera were kept at 4 °C before use.

Enzyme-linked immunosorbent assay. The reactivity of mouse and rabbit antisera or anti-RBD mAbs with S proteins (RBD-Fc, RBD-R441A or FL-S) was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 1 μg/ml recombinant protein was used to coat 96-well microtiter plates (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, serially diluted antisera or mAbs were added and incubated at 37 °C for 1 h, followed by four washes with PBS containing 0.1% Tween 20. Bound antibodies were detected with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Zymed) at 37 °C for 1 h, followed by washes. The reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) and absorbance at 450 nm was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC).

Generation of SARS pseudovirus and neutralization assay. SARS-CoV pseudovirus system was developed in our laboratory as previously described [27,28]. In brief, HEK293T cells were co-transfected with a plasmid encoding the S protein corresponding to SARS-CoV Tor2 isolate and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE) by using Fugene 6 reagents (Boehringer Mannheim). Supernatants containing SARS pseudovirus were harvested 48 h post-transfection and used for single-cycle infection of human ACE2-transfected 293T (293T/ACE2) cells. Briefly, 293T/ACE2 cells were plated at 10^4 cells/well in 96-well tissue-culture plates and grown overnight. The supernatants containing pseudovirus were pre-incubated with serially diluted mouse or rabbit antisera at 37 °C for 1 h before addition to cells. The culture was re-fed with fresh medium 24 h later and incubated for an additional 48 h. Cells were washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well Costar flat-bottomed luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega). Relative light units (RLU) were determined immediately in the Ultra 384 luminometer (Tecan US).

Receptor-binding assays. Binding of RBD-Fc or RBD-R441A protein to soluble ACE2 was measured by ELISA. Briefly, recombinant soluble ACE2 (R&D Systems, Inc., Minneapolis, MN) at 2 μg/ml was coated onto 96-well ELISA plates (Corning Costar) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, serially diluted RBD-Fc or R441A was added to the wells and incubated at 37 °C for 1 h. After washing, the HRP-conjugated goat anti-human IgG (Zymed) was added and incubated an additional 1 h. After washing, the substrate TMB was used for detection. Binding of RBD-Fc or RBD-R441A to ACE2-expressing cells was measured by flow cytometry. Briefly, 10^6 293T/ACE2 cells were detached, collected, and washed with Hanks' balanced salt solution (HBSS) (Sigma, St. Louis, MO). RBD-Fc or RBD-R441A was added to the cells to a final concentration of 1 μg/ml, followed by incubation at room temperature for 30 min. Cells were washed with HBSS

and incubated with anti-human IgG-FITC conjugate (Zymed) at 1:50 dilution at room temperature for an additional 30 min. After washing, cells were fixed with 1% formaldehyde in PBS and analyzed in a Becton FACSCalibur flow cytometer (Mountain View, CA) using CellQuest software.

Results

Amino acid residue 441 (arginine) is essential for the immunogenicity of RBD to induce neutralizing antibodies

It was recently reported that the full-length S protein of SARS-CoV bearing R441A substitution failed to induce neutralizing antibodies against S protein-pseudotyped viruses [33]. We are interested in investigating whether this point mutation can also affect the immunogenicity of RBD-Fc, a potent inducer of neutralizing antibodies [28,29]. The RBD-R441A mutant was generated by mutagenesis as described. Both wild-type RBD-Fc and RBD-R441A were expressed in 293T cells and purified to homogenicity by protein A chromatography (Fig. 1C). For comparison, both the wild-type and mutant proteins were, respectively, used to immunize mice and rabbits. As shown in Fig. 2, both mice and rabbits developed robust antibody responses against the corresponding immunogens after the third boosting immunizations. Relatively, RBD-Fc induced higher titers of antibodies in both mice (mean end-point titer was 1/656,100) and rabbits (mean end-point titer was 1/5,904,900), whereas RBD-R441A induced antibodies with mean end-point titers at 1/145,800 in mice and at 1/328,050 in rabbits. We used a recombinant full-length S protein (FL-S) as a coating antigen to measure the titers of antibodies specific for the RBD in the antisera collected after the third boost (Fig. 3). Surprisingly, while the RBD-Fc could induce high titers of RBD-specific antibodies in mice and rabbits (mean end-point titers were 1/656,100 and 1/437,400, respectively), the R441A mutant only induced RBD-specific antibody titers at 1/2700 in mice and 1/1800 in rabbits, respectively.

We then tested whether mouse and rabbit antisera had neutralizing activities against S protein-mediated viral entry by using SARS pseudovirus. Consistent with our previous findings [28,29], both mouse and rabbit antibodies induced by RBD-Fc could potently neutralize SARS pseudovirus with mean 50% neutralizing titers at 1/58,320 and 1/19,440, respectively (Fig. 4). However, the antibodies induced by RBD-R441A mutant in both mice and rabbits could not significantly inhibit SARS pseudovirus at 1/40 or higher dilutions. These results indicate that R441A substitution severely impairs the immunogenicity of RBD-Fc to elicit RBD-specific neutralizing antibodies in immunized animals.

R441A substitution determines the antigenic structure in the RBD of S protein

We previously identified six groups of conformation-dependent neutralizing epitopes (Conf I–VI) with a panel

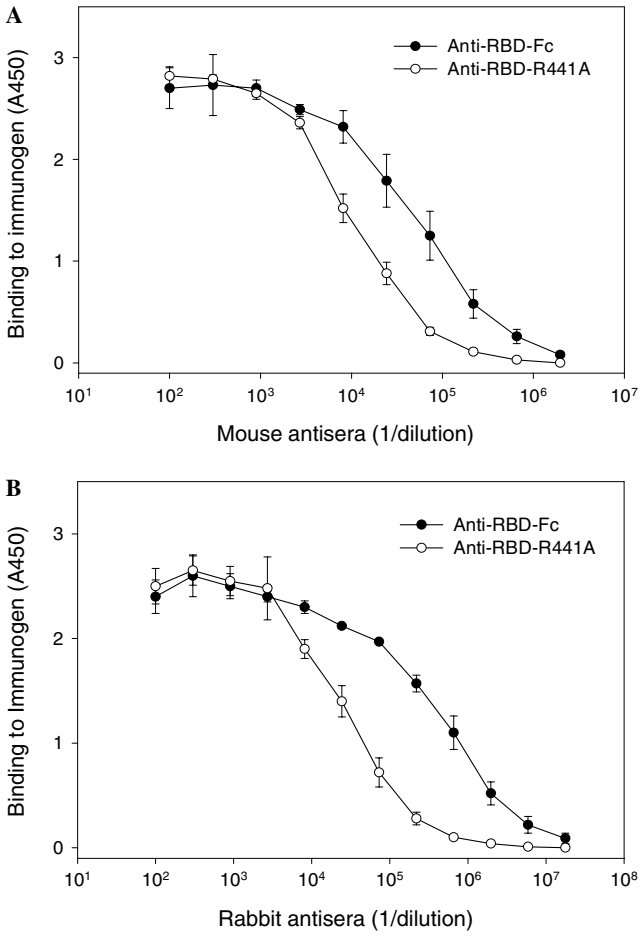

Fig. 2. Antibody responses in mice (A) and rabbits (B) immunized with RBD-Fc or RBD-R441A. Binding of serially diluted mouse or rabbits antisera collected after the third boost to the corresponding immunogen was measured by ELISA.

of 27 anti-RBD mAbs isolated from the mice immunized with RBD-Fc [29]. We have recently isolated a set of novel anti-RBD mAbs from the mice immunized with inactivated SARS-CoV or recombinant full-length S protein, and their epitopes have been initially grouped by binding competition (designated as Group A–C and Group a–c, respectively) (Fig. 5). Consistently, these novel anti-RBD mAbs possess potent neutralizing activity, but they may target different epitopes as shown by their unique specificity to block receptor binding (data not shown). To probe antigenic structure in the RBD bearing R441A mutation, the representative anti-RBD mAbs from each epitopic groups were used in ELISA to measure their reactivity with both wild-type and mutant proteins. As shown in Fig. 5, all anti-RBD mAbs strongly reacted with RBD-Fc, but only three conformation-dependent mAbs (24F4, 33G4, and S25) were able to recognize the RBD-R441. We have known that the epitope for S25 (Group D) differs from those of the Conf V mAbs (24F4 and 33G4) as shown by their binding competition and their capacity to block receptor binding (data not shown). It was of interest to note that the reactivity of one mAb (4D5) targeting the

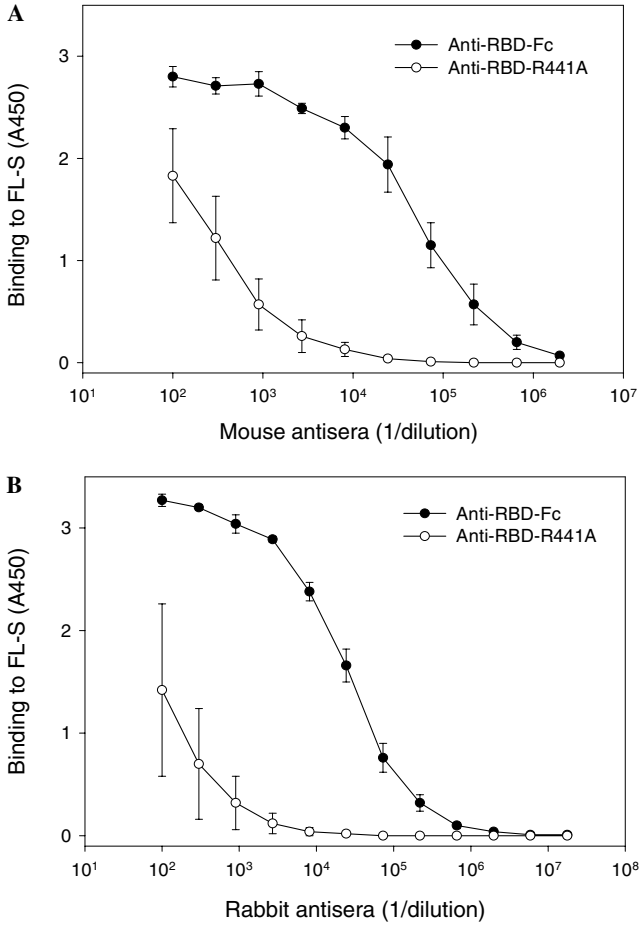

Fig. 3. Binding titers of mouse (A) and rabbit (B) antisera to the full-length S protein (FL-S) measured by ELISA.

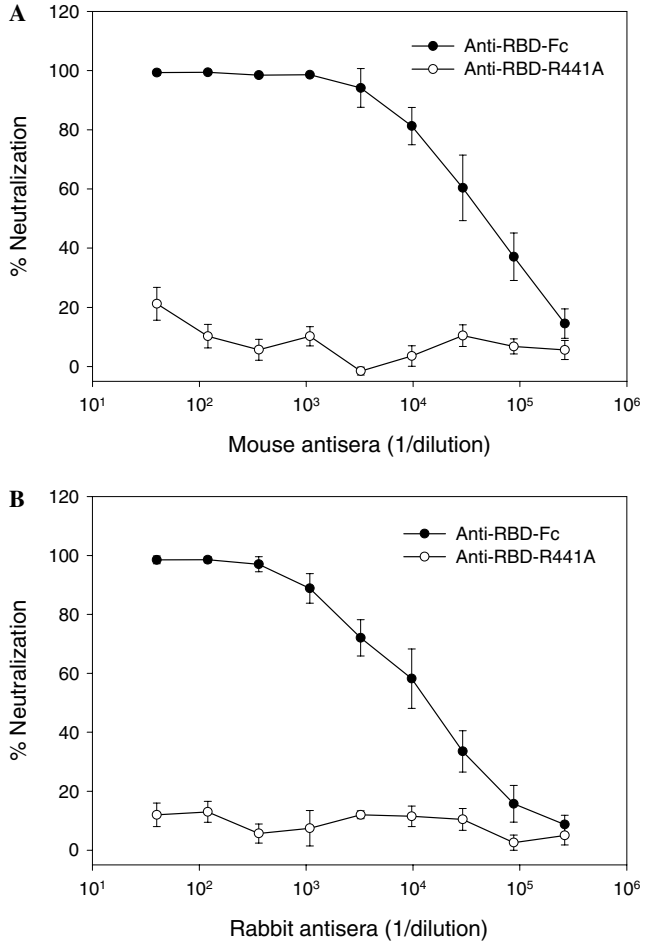

Fig. 4. Neutralizing activity of mouse (A) and rabbit (B) antisera against SARS pseudoviruses. Infection of HEK293 cells expressing human ACE2 by SARS pseudoviruses (Tor2) was determined in the presence of mouse or rabbit antisera at a series of 3-fold dilutions. Percent neutralization was calculated for each sample and the average values were plotted.

linear epitope (residues 435–451) was significantly reduced by R441A substitution, while the mAb 17H9 targeting the residues 458–465 reacted with both proteins equally. These results suggest that R441A substitution can disrupt the majority of conformation-dependent neutralizing epitopes in the RBD, and that the Conf V and Group D epitopes are relatively stable and conserved.

To further characterize the antigenicity of RBD-R441A mutant, we compared it with wild-type RBD-Fc for the reactivity with the mouse antisera induced against the FL-S by ELISA. As expected, the RBD-Fc reacted strongly with the mouse anti-S sera (mean end-point titer at 1/218,700), comparable with the FF-S protein (1/328,050) (Fig. 6). However, RBD-R441A mutant did not significantly react with mouse anti-FL-S sera, confirming that R441A mutation disrupts the antigenic structure in the RBD.

R441A substitution abolishes the interaction of RBD with the receptor ACE2

The S protein of SARS-CoV is responsible for binding with the receptor and mediates viral entry into target cell [7]. However, pseudovirus expressing the full-length S protein with R441A substitution completely loses infectivity [33]. We hypothesized that the substitution of R441 might disrupt the receptor-binding motif in the RBD and thereby the pseudovirus was unable to bind to the receptor. First, we used an ELISA-based assay to compare the binding activities of both RBD-Fc and RBD-R441A to soluble ACE2. As shown in Fig. 7, RBD-Fc could bind to the soluble ACE2 in a dose-dependent manner, whereas RBD-R441A had no binding activity at a concentration up to 10 μg/ml, at which the wild-type RBD-Fc reached a reactive plateau. We then measured whether RBD-Fc and RBD-R441A bind to cell-associated ACE2 by flow cytometry. Consistent with our previous report, RBD-Fc bound to ACE2-expressing 293T cells efficiently; however, the RBD-R441A could not bind to ACE2 expressed on the cells (Fig. 8).

RBD-Fc itself had inhibitory activity against pseudovirus entry, suggesting its potential application as an antiviral therapeutic [14]. In parallel, we tested the inhibitory activity of both RBD-Fc and RBD-R441A on SARS pseudovirus. As expected, RBD-Fc was able to inhibit SARS pseudovirus infection with an IC_{50} of 1.12 μg/ml

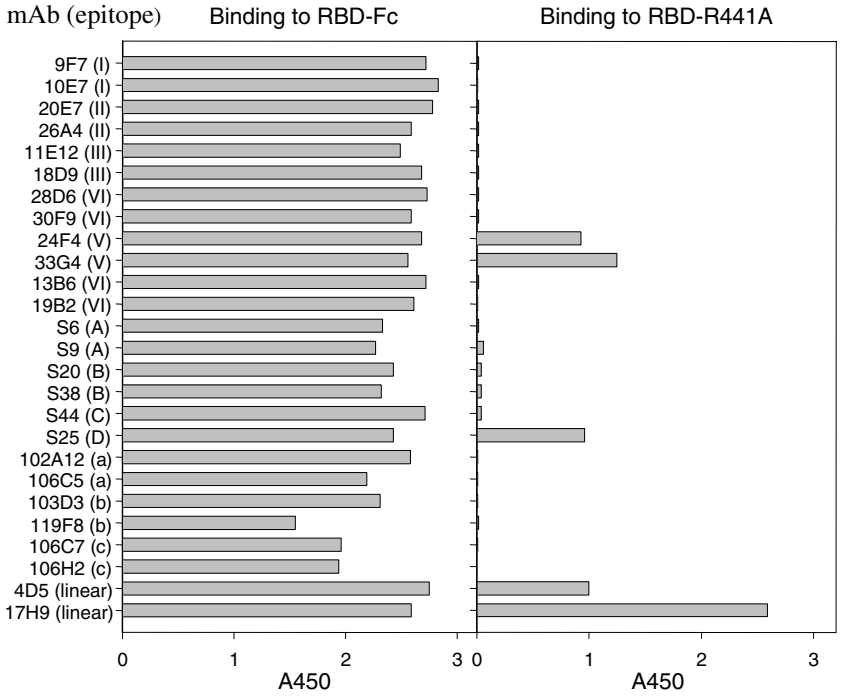

Fig. 5. Reactivity of RBD-Fc and RBD-R441A mutant with anti-RBD mAbs that recognize different epitope conformations in RBD measured by ELISA. Antigens were coated to ELISA plates at 1 μg/ml and mAbs were tested at 10 μg/ml.

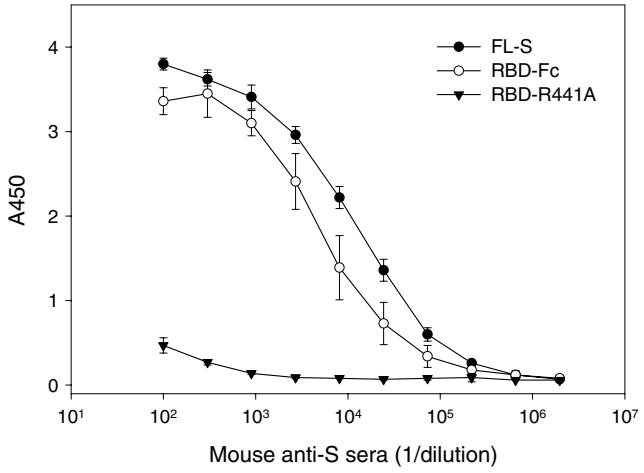

Fig. 6. Reactivity of RBD-Fc and RBD-R441A mutant with mouse anti-S antisera measured by ELISA. Antigens (FL-S, RBD-Fc, and RBD-R441A) were coated to ELISA plates at 1 μg/ml and mouse antisera were tested at 3-fold dilutions.

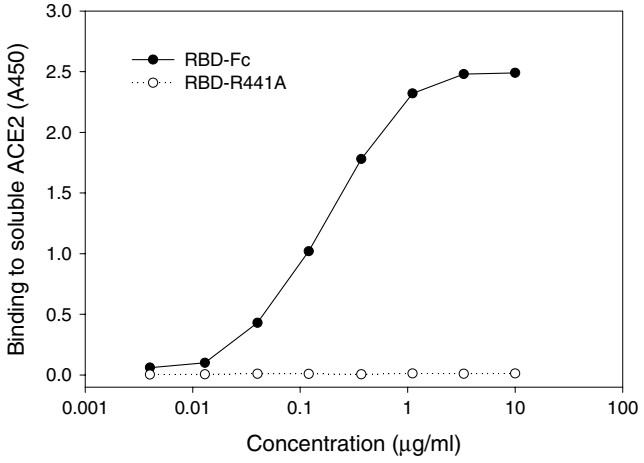

Fig. 7. Binding activity of RBD-Fc and RBD-R441A mutant to soluble ACE2. Soluble ACE2 was coated to plates at 2 μg/ml and binding of serially diluted RBD-Fc or R441A was measured by ELISA.

(Fig. 9), whereas the RBD-R441A had no inhibitory effect at a concentration as high as 100 μg/ml, suggesting that the RBD mutant could not compete with the virion to bind to the receptor. Taken together, these data suggest that R441A substitution may damage the receptor-binding motif in the RBD.

Discussion

SARS-CoV emerged in the winter of 2002–2003 and killed approximately 800 people, ~10% of those infected [1,2,34–36]. Although there are no recent SARS outbreaks, the need to develop effective vaccines remains of high importance to prevent future epidemic caused by the SARS-CoV, which may re-emerge from animal reservoirs [37–41]. To this end, structural and functional characterization of SARS-CoV is one of the highest priorities. Yi et al. [33] recently reported that single amino acid substitutions in the SARS-CoV S protein determine the viral entry and immunogenicity to induce neutralizing antibodies, but the mechanisms that caused these phenotypes remain to be elucidated. In this study, we used the RBD-Fc as a model

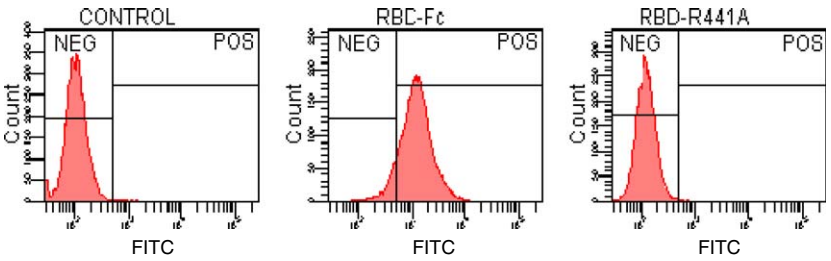

Fig. 8. Binding activity of RBD-Fc and RBD-R441A mutant to cell-associated ACE2. Binding of RBD-Fc and RBD-R441A mutant at 1 μg/ml to the HEK293 cells expressing human ACE2 was measured by flow cytometry.

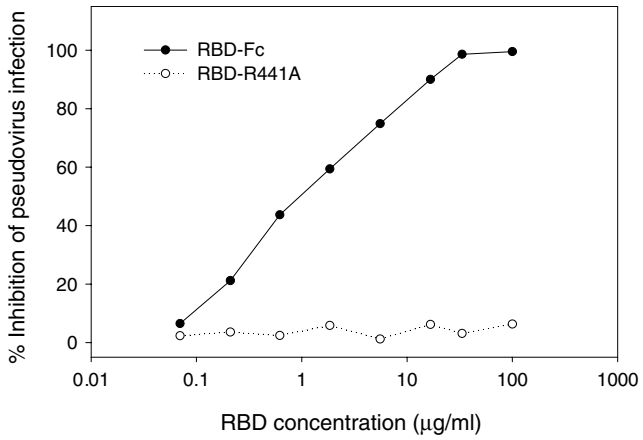

Fig. 9. Inhibitory activity of RBD-Fc and RBD-R441A mutant against SARS pseudoviruses. Infection of HEK293 cells expressing human ACE2 by SARS pseudoviruses (Tor2) was determined in the presence of RBD-Fc or RBD-R441A at a series of 3-fold dilutions. Percent inhibition was calculated for each sample.

to study how a single residue mutation in the RBD can abolish the major function of full-length S protein, since this molecule can efficiently bind to the receptor ACE2 and contains multiple conformation-dependent epitopes (Conf I–VI) capable of inducing highly potent neutralizing antibodies [29]. We converted arginine 441 to alanine (R441A), which was shown to disrupt the immunogenicity of full length to induce neutralizing antibodies and S protein-mediated viral entry [33], and evaluated its effect on the antigenic structure and binding function of the RBD. First, we found that R441A substitution could completely abolish the ability of RBD to induce neutralizing antibodies in the immunized mice and rabbits. We then probed the antigenic epitopes in the RBD bearing R441A by a panel of anti-RBD mAbs recognizing different epitopes in the RBD and found that this mutation could disrupt the major neutralizing epitopes. These results provide direct evidence to explain why RBD-R441A mutant could not induce neutralizing antibodies. Although Conf V and Group D neutralizing epitopes in the RBD-R441A retained partial reactivity with the corresponding mAbs, they failed to elicit functional antibodies in either mice or rabbits. These data indicate that the residue R441 is essential for maintaining the antigenic structure in the RBD, which confers the immunogenicity to induce neutralizing antibodies.

It was understandable that a single residue change in the RBD (e.g., R441A) could abolish its ability to induce functional antibodies through disrupting its major conformation-dependent neutralizing epitopes, but the mechanism how R441A substitution was able to determine the immunogenicity of full-length S protein is poorly understood [15]. Although the RBD of SARS-CoV is a major target of neutralizing antibodies, the S protein also contains some neutralizing epitopes in the other domains. For example, it was reported that the linear epitopes in the HR2 region of the S2 subunit, which is far away from the RBD, could induce antibodies with moderate neutralizing activity [30]. We have recently found that the N-terminal region (residues 13–327) of S protein also contains neutralizing epitopes (data not shown). Therefore, further structural characterization on the S protein may provide important information for understanding why a single point mutation in RBD affects the immunogenicity of the entire S protein.

We subsequently documented that R441A mutation was able to completely abolish RBD-mediated binding activity to the receptor ACE2. The RBD-R441A molecule could not bind to either soluble or cell-associated ACE2 as shown by ELISA and flow cytometry-based assays, respectively. Moreover, the RBD with R441A substitution also lost its inhibitory ability against viral entry. Therefore, our data indicate that the residue R441 is not only essential for the antigenic structure in the RBD, but also critical for the receptor-binding motif (RBM). Crystal structure of the RBD in complex human ACE2 reveals that only a few of the many contacting residues in the large interface between the S protein and receptor determine the efficiency of virus binding and infection [29]. The ACE2 is bound by an extended loop in the S protein that projects from a compact core formed by residues 424–494, the RBM. In particular, a methyl group from a threonine residue at position 487 of the S protein at the interface extends into a hydrophobic pocket in ACE2 that contains a lysine residue at position 353. Although the R441 is not one of 14 residues on the loop that contact 18 residues on human ACE2 [29], its substitution might change the configuration of RBM and thereby disrupts interaction of the residues between RBM and ACE2. Subsequently, the conformational change of the RBD might result in a dramatic alteration in its antigenic structure. Therefore, retaining the critical residues and proper antigenic conformations in RBD is

important for developing SARS vaccines. The sites in RBD containing critical residues, e.g., R441, can be used as targets for rational design of therapeutics.

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