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Single Amino Acid Substitutions in the Severe Acute Respiratory Syndrome Coronavirus Spike Glycoprotein Determine Viral Entry and Immunogenicity of a Major Neutralizing Domain

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Neutralizing antibodies (NAbs) against severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) spike (S) glycoprotein confer protection to animals experimentally infected with the pathogenic virus. We and others previously demonstrated that a major mechanism for neutralizing SARS-CoV was through blocking the interaction between the S glycoprotein and the cellular receptor angiotensin-converting enzyme 2 (ACE2). In this study, we used in vivo electroporation DNA immunization and a pseudovirus-based assay to functionally evaluate immunogenicity and viral entry. We characterized the neutralization and viral entry determinants within the ACE2-binding domain of the S glycoprotein. The deletion of a positively charged region SΔ(422-463) abolished the capacity of the S glycoprotein to induce NAbs in mice vaccinated by in vivo DNA electroporation. Moreover, the SΔ(422-463) pseudovirus was unable to infect HEK293T-ACE2 cells. To determine the specific residues that contribute to related phenotypes, we replaced eight basic amino acids with alanine. We found that a single amino acid substitution (R441A) in the full-length S DNA vaccine failed to induce NAbs and abolished viral entry when pseudoviruses were generated. However, another substitution (R453A) abolished viral entry while retaining the capacity for inducing NAbs. The difference between R441A and R453A suggests that the determinants for immunogenicity and viral entry may not be identical. Our findings provide direct evidence that these basic residues are essential for immunogenicity of the major neutralizing domain and for viral entry. Our data have implications for the rational design of vaccine and antiviral agents as well as for understanding viral tropism.

Severe acute respiratory syndrome (SARS), first identified in China in November 2002, is an emerging infectious disease caused by a novel coronavirus (CoV) variant, SARS-CoV (11, 22, 23, 26). SARS-CoV is highly transmissible in humans, with a mortality rate near 10% (10, 25, 28). Because of the threat of a reemerging epidemic, much effort has been placed on the development of a prophylactic vaccine against the pathogenic SARS-CoV (3, 5, 6, 20, 33, 36). Initially, it was first shown in a mouse model that passively transferred antibodies can prevent SARS-CoV replication in the lung following intranasal viral challenge (29). Subsequently, several groups reported that DNA and live virus (adenovirus type 5 and modified vaccinia Ankara [MVA]) vaccines expressing SARS-CoV spike (S) glycoprotein were able to induce T-cell and antibody responses (3, 6, 12, 36, 39, 41, 43). Moreover, protective immunity was achieved using a DNA or MVA vaccine in a mouse model (3, 36). Further observations show a correlate of protection mediated by neutralizing antibodies (NAbs) (3, 36). These findings are encouraging and indicate the potential for vaccine-induced protective immunity in humans.

Shortly after SARS-CoV was identified, Li et al. revealed that angiotensin-converting enzyme 2 (ACE2) was the functional receptor for SARS-CoV (24). The interaction between ACE2 and SARS-CoV S glycoprotein was further elucidated to reveal the structure and function of ACE2 and of the viral envelope protein (27). ACE2 was found to interact with an independently folded receptor binding domain (RBD), a 193-residue fragment [S(318-510)]) of the SARS-CoV S protein (34). This 193-residue fragment alone exhibited potent antiviral activity through blocking S-protein-mediated infection. This finding suggests that the functional elements required to compete with SARS-CoV binding to ACE2 are located within amino acids 318 to 510. In a separate study, a single-chain variable fragment 80R human monoclonal antibody (MAb) efficiently neutralized SARS-CoV and inhibited syncytia formation between cells expressing the S protein and those expressing ACE2 (30). Since this antibody also blocked the interaction between ACE2 and S glycoprotein, the ACE2-binding site of the S glycoprotein has become an attractive target for vaccine design (30).

Of the structural proteins that compose SARS-CoV, the S glycoprotein is probably the only significant target for neutralization (4). We and others previously demonstrated that the receptor binding domain of the S protein contains a major neutralization determinant (6, 15, 16), which can induce potent NAbs that block SARS-CoV replication in monkeys (6). Regardless of the forms of vaccines tested (e.g., DNA, MVA, and inactivated virus) (3, 16, 35, 36), a major neutralization determinant is well exposed in vivo for inducing substantial levels of NAbs. However, specific mapping of the major neutralizing epitope is yet to be determined. Here, we delete a mainly positively charged region, SΔ(422-463), in the receptor binding domain of the S glycoprotein and observe a loss in the ability

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to induce potent NAbs as well as mediate viral entry. Moreover, these phenotypes are determined by positively charged residues in the deletion region. Our findings have implications for SARS vaccine and antiviral design in addition to understanding the biological function of the viral envelope glycoprotein.

MATERIALS AND METHODS

SARS-CoV S glycoprotein mutagenesis. The codon-optimized full-length SARS-CoV S gene was cloned into pcDNA3.1 as a positive control (pcDNA3.1-OPT9-S), as previously described (6). Based on pcDNA3.1-OPT9-S, we created a construct, pCMV-SΔ(422-463), by removing a 42-amino-acid (aa) region, Δ(422-463), within the ACE2-receptor binding region of the S gene. Additionally, primers were constructed to mutate eight basic residues, R426, K439, R441, R444, H445, K447, R449, and R453, to alanine. A total of eight constructs (mutants 1 through 8) was derived from the codon-optimized full-length S gene of SARS-CoV in pcDNA3.1 (Invitrogen, Carlsbad, CA), using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Each of the mutated residues lies within the deletion region of pCMV-SΔ(422-463). Each plasmid was purified with the GenElute Endotoxin-free Plasmid Maxiprep kit (Sigma-Aldrich, St. Louis, MO) and resuspended in saline. All plasmids were confirmed by sequence analysis through the DNA Sequencing Core Facility at the Aaron Diamond AIDS Research Center.

Expression of mutant S proteins. 293T cells (5×10^5 cells per well) in 6-well plates were transfected (Superfect Transfection Reagent; QIAGEN, Valencia, CA) with 2 μg of pcDNA3.1-OPT9-S, pCMV-SΔ(422-463), or pcDNA3.1 mutants 1 through 8. Forty-eight hours posttransfection, cells were lysed on ice for 30 min in 100 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 137 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% glycerol, 1 μg/ml each of pepstatin, leupeptin, and pefabloc), cleared of lysate (14,000 rpm for 10 min, 4°C), boiled at 100°C for 15 min, and run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen). After transfer to polyvinylidene difluoride membrane (Invitrogen), blots were blocked in 5% milk and 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), washed, and incubated with rabbit 419 (R419D57) serum (1:100) in blocking buffer. R419D57 contains polyclonal antibodies to the first 400 amino acids of the N-terminal region of the S protein, as previously described (6). Blots were washed and incubated with protein-G horseradish peroxidase (HRP) conjugate (1:4,000; Bio-Rad, Hercules, CA). Immunofluorescence was measured with ECL plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ). In a parallel study using immunochemical staining, 48 h posttransfection cells were washed with PBS and fixed with 100% cold methanol for 10 min at −20°C. Fixed cells were blocked with 1% BSA in PBS, washed, and incubated with R419D57 serum (1:100) for 1 h at 37°C. Following primary antibody incubation, cells were washed and incubated with anti-rabbit immunoglobulin (Ig)-HRP (1:2,000; Amersham Biosciences). A similar method was used to test mice sera immunized with mutant 3, mutant 8, OPT9, and SΔ(422-463). HEK-293T cells transfected with pcDNA3.1-OPT9-S were stained with mouse immune sera (1:50), washed, and incubated with anti-mouse Ig-HRP (1:2,000; Amersham Biosciences). Color detection was determined with DAB Enhanced Liquid Substrate System (Sigma-Aldrich) working solution.

Animal immunizations. Ten groups (2 mice per group) of 6- to 8-week-old female BALB/c mice (Charles River Laboratories) were immunized with 20 μg of plasmid DNA in 50 μl saline by in vivo electroporation (EP) (ICHOR MedSystem, San Diego, CA). Mice were vaccinated every 3 weeks, and sera were collected at weeks 2 and 5. Two mice immunized with mutant 3 (R441A) were later boosted with two additional injections of pcDNA3.1-OPT9-S at weeks 6 and 9, and sera were collected at weeks 8 and 11. An equivalent study was conducted on three groups of mice (2 mice per group) immunized with pcDNA3.1-OPT9-S and R441A mutant for measurement of cytotoxic T-lymphocyte responses. Serum from this study was collected at weeks 2 and 5. At the end of the study (week 5), mice were euthanized and spleens were collected. All animal experiments were approved by and conducted in the Laboratory Animal Research Center at the Rockefeller University.

Indirect enzyme-linked immunosorbent assay (ELISA). Costar EIA/RIA high binding 96-well flat-bottom plates (Corning, Inc., Corning, NY) were coated with 0.5 μg/well affinity-purified anti-6× His tag antibodies (rabbit) (Rockland, Gilbertsville, PA) in coating buffer (pH 9.6, 0.1 M $NaHCO_3$) at 4°C overnight. The plates were washed four times with PBST (PBS containing 0.05% Tween-20) and blocked with 5% nonfat milk–0.5% BSA in PBS at 37°C for 2 h. S400 and S600 proteins, which have 6× His tags at their C terminals (6), were then captured by anti-His antibody when 200 μl/well supernatant of 293T cells transfected with plasmids encoding proteins S400 or S600 was incubated at 37°C for 1 h. After washing, serial-diluted (1:3) mouse immune sera was added and incubated at 37°C for 1 h. After washing, 1:5,000 diluted anti-mouse Ig-horseradish peroxidase (sheep) (Amersham Biosciences) was incubated at 37°C for 1 h. After the last wash, the color was developed with substrate solution and the optical density at 450 nm was measured. Values twofold above the background control were considered positive.

Cell-mediated immune responses measured by gamma interferon ELISpot assays. Cell-mediated immune responses were quantified using a commercially available murine interferon-ELISpot assay (R&D systems, Minneapolis, MN). Briefly, 96-well nitrocellulose-backed plates (MAHA S45; Millipore, Billerica, MA) were coated with capture antibodies (Abs) (R&D Systems) in coating buffer (pH 9.6, 0.1 M $NaHCO_3$) at 4°C overnight. The plates were washed four times with PBST (PBS containing 0.05% Tween-20) and blocked with R10 (RPMI 1640 containing 10% fetal calf serum, antibiotics, and 2 μl β-mercaptoethanol in a 500-ml volume, 200 μl/well) at 37°C for 2 h. Splenocytes were isolated from mice immunized with R441A mutant (2 mice) and pcDNA3.1-OPT9-S (1 mouse) on week 4. Freshly prepared mouse splenocytes were added (either 0.5×10^6 or 1.0×10^6 cells/well), followed by SARS-CoV receptor binding region-Fc fusion protein (1 μg/well) (6). *Staphylococcus aureus* enterotoxin B was used as a positive control, whereas no protein and purified recombinant human immunodeficiency virus type 1 (HIV-1) Gag-Fc was used as a negative control. The cells were incubated overnight at 37°C and washed five times with PBST. The detection Ab was added, and the plates were incubated overnight at 4°C. After washing with PBST, streptavidin-alkaline phosphatase was added at room temperature for 2 h. The plates were washed four times with PBST and once with distilled H_2O. Finally, 100 μl of substrate (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium chromogen) was added to each well and incubated in the dark at room temperature for 15 min. The plates were rinsed with tap water and allowed to dry thoroughly. Blue spots were counted by using an ELISpot reader (ImmunoSpot; Cellular Technology Ltd., Cleveland, OH).

Pseudovirus entry assay. The pseudovirus was generated by cotransfecting (Superfect; QIAGEN) 293T cells with pNL4-3Luc$^+$Env$^-$Vpr$^-$ and either pcDNA3.1-OPT9-S, pCMV-SΔ(422-463), or pcDNA3.1 mutants 1 through 8. Cell supernatant was collected 48 h posttransfection and frozen at −150°C. p24 was measured (HIV-1 p24 Antigen EIA; Beckman Coulter, Fullerton, CA) by the Aaron Diamond AIDS Research Center Virology Core Laboratory. HEK293T-ACE2 or NIH 3T3/MX-L-SIGN cells (10,000 cells per well in 100 μl) were plated in 96-well plates at 37°C overnight. The NIH 3T3/MX-L-SIGN cell line was purchased from the National Institutes of Health AIDS Reagent Depository (catalog no. 9948). On the following day, 10 ng (based on p24 concentrations) of pseudotype virus and 16 ng polybrene (in 100 μl medium) were added to the cells. Seventy-two hours after infection, cells were washed with PBS and lysed (1× Cell Culture Lysis Reagent; Promega, Madison, WI). Thirty microliters of cell lysate was mixed with 100 μl of Luciferase Assay Reagent (Promega), and luciferase intensity was measured.

Neutralization assay. A pseudovirus-based neutralization assay was established to determine the humoral immune responses against SARS-CoV (6, 7). The neutralizing activity of heat-inactivated sera (56°C, 30 min) was determined by mixing 10 ng of pseudotype virus (in 30 μl) with diluted serum (in 30 μl) at 37°C for 1 h. After neutralization, the mixture was combined with 16 ng polybrene (in 40 μl medium) and added to HEK293T-ACE2 or 786-O cells (10,000 cells per well in 100 μl). The 786-O cell line was purchased from the American Type Culture Collection (ATCC CRL-1932). Cells were washed with PBS and lysed (1× Cell Culture Lysis Reagent; Promega) 56 to 72 h after infection. Luciferase intensity was measured, and the percentage of neutralization was calculated.

RESULTS

A deletion within the receptor binding region of the S glycoprotein abolishes immunogenicity of the major neutralizing domain and viral entry. In our previous study, we mapped a major neutralizing antibody determinant between residues 400 and 600 at the N-terminal region of the S glycoprotein (6). This region not only serves as a dominant B-cell NAb epitope but also overlaps with the ACE2 receptor binding domain [RBD; residues 318 to 510] (34). Further analysis of this epitope revealed eight basic amino acids (R426, K439, R441, R444,

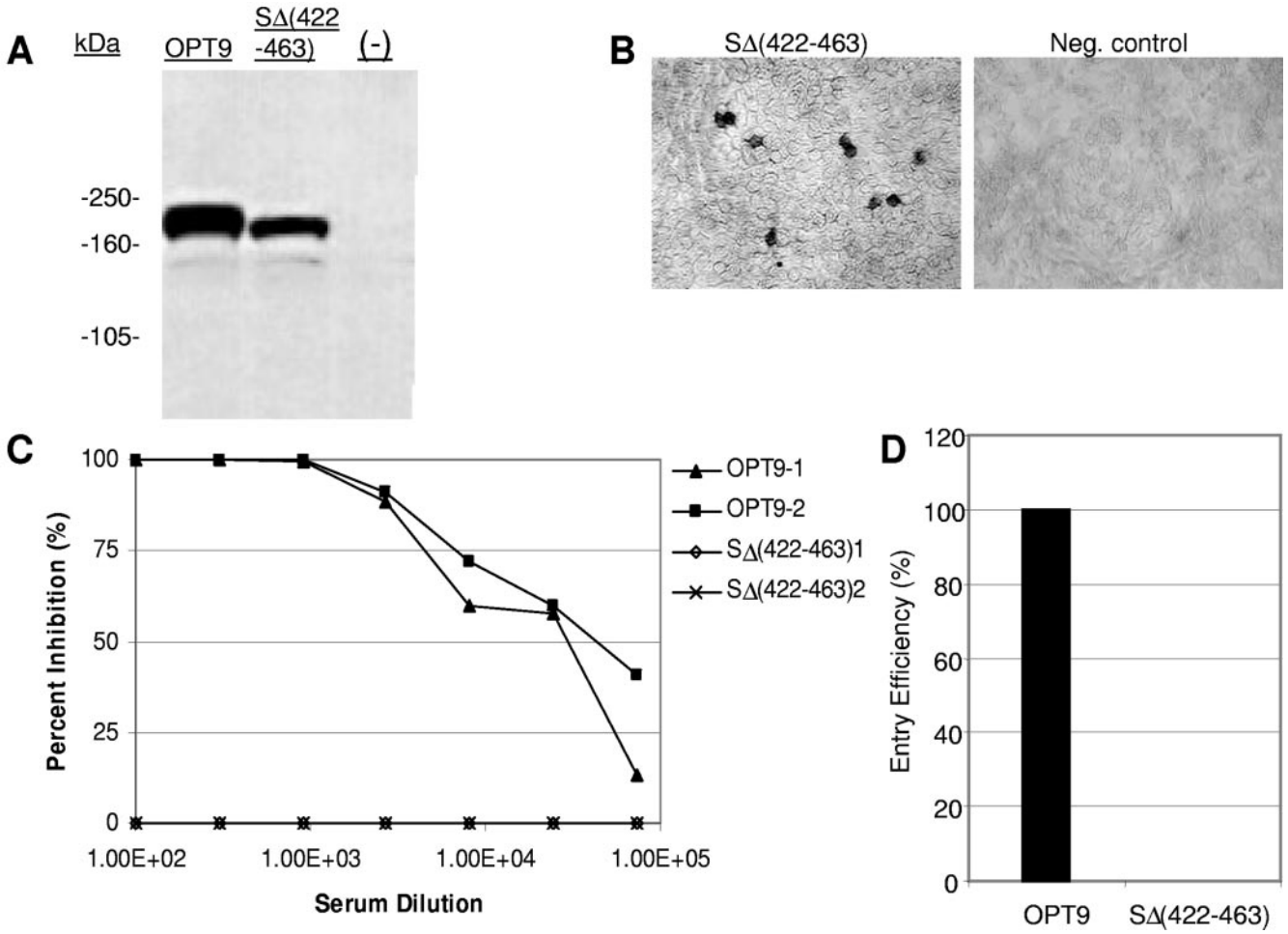

FIG. 1. Comparison of full-length S (OPT9) glycoprotein versus the deletion SΔ(422-463) mutant. (A) Western blot expression of the full-length codon-optimized (OPT9) S glycoprotein (left), mutant SΔ(422-463) (middle), and 293T cell lysate (right). (B) 293T cell surface staining of mutant SΔ(422-463) expression (left) and 293T cells (right). Expression was measured with a polyclonal antibody to the first 400 amino acids of the S glycoprotein at a 1:100 dilution. (C) Neutralization assay of mice sera immunized with two injections of OPT9 or the mutant SΔ(422-463). Mutant SΔ(422-463) failed to induce neutralization activity. (D) Entry assay of the OPT9 set at 100% efficiency, compared with the lack of entry with mutant SΔ(422-463) pseudotyped virus. Neg., negative.

H445, K447, R449, and R453) identified in a region [S(420-466)] of the S glycoprotein. Since the positively charged residues correspond with the highly negatively charged surface of the cellular receptor ACE2 (27), we hypothesized that electrostatics may play an important role in the interaction between the S protein and ACE2 receptor. To test this hypothesis, we replaced the target region of 42 amino acids (residues 422 to 463) with 7 neutral spacer amino acids (GSGGGLE). This mutant S protein, called SΔ(422-463), was derived from the codon-optimized full-length S gene used in our parental DNA vaccine (pcDNA3.1-OPT9), as previously described (6). By Western blot analysis and cell surface staining, we confirmed that SΔ(422-463) is properly expressed in 293T-transfected cells. Protein levels were determined by immunostaining with rabbit R419D57 serum raised against the first 400 amino acids of the S protein (Fig. 1A and B). To determine the immunogenicity of the SΔ(422-463) mutant, we immunized mice with either our SΔ(422-463) mutant or pcDNA3.1-OPT9 parental plasmid DNA through in vivo electroporation (EP). This technique enhanced the generation of antibody response by more than 100-fold in comparison to intramuscular injection of naked DNA. It also gave a consistent antibody response in every vaccinated animal (unpublished data). Serum was collected after two injections and was measured for the ability to neutralize our pseudovirus, OPT9 (6). We failed to detect the presence of NAbs after two immunizations with the SΔ(422-463) mutant (<1:10 serum dilution), although binding antibodies were observed. In contrast, mice immunized with pcDNA3.1-OPT9 produced substantially higher levels of NAbs (50% inhibitory concentration, >1:38,000 serum dilution) (Fig. 1C). These results indicate that the deleted region Δ(422-463) contains essential elements for inducing major NAbs. To examine the functional determinants necessary for the deletion region and ACE2 receptor interaction, the SΔ(422-463) mutant plasmid was used to generate pseudovirus expressing luciferase as a reporter gene. The SΔ(422-463) pseudovirus was subsequently used to infect HEK293T-ACE2 cells. As shown in Fig. 1D, SΔ(422-463) pseudovirus failed to infect cells when

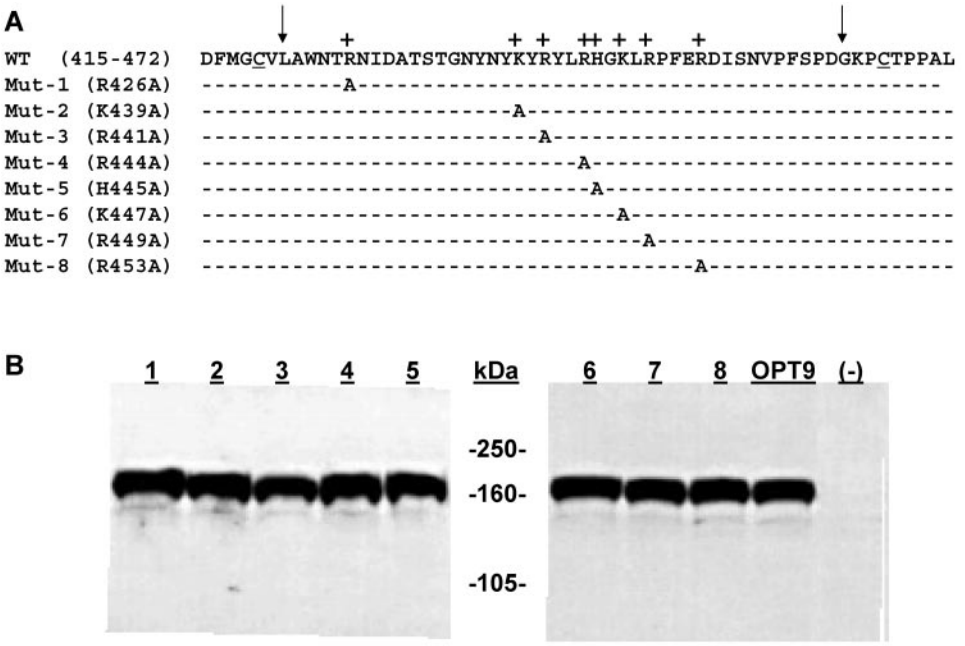

FIG. 2. Construction of eight individual S glycoprotein point mutations and comparative expression. (A) Schematic diagram of the ACE2 receptor binding region with arrows indicating the residue outside the SΔ(422-463) deletion region. The eight positively charged residues are annotated (+) above each corresponding residue, and the individual mutations are shown below as a change to an alanine. (B) Similar levels of expression of each of the mutants (1 to 8), OPT9, and cell lysate (−) are detected in Western blots stained with a polyclonal antibody to the first 400 amino acids of the N terminus. WT, wild-type; Mut-, mutant.

measured against the control pseudotype virus (OPT9), suggesting the loss of entry determinants. Our findings also provide new evidence that the ACE2 receptor binding region coincides with a dominant neutralization domain in the S glycoprotein of SARS-CoV.

Amino acid R441 determines the immunogenicity of a major neutralizing domain in the S glycoprotein. Since the deletion may artificially disrupt the core structure of the receptor binding region, we further determined the impact of individual residues on immunogenicity and viral entry. Because the S(422-463) region contains a significant number of positively charged residues (9 out of 47), we sought to determine the role of these basic residues in inducing high levels of NAbs. We generated eight mutants derived from pcDNA3.1-OPT9 by replacing each of the positively charged residues with alanine (Fig. 2A). We did not mutate residue K465 because it is located outside the deleted region. Immunoblotting (Fig. 2B) and immunostaining (data not shown) were conducted to confirm proper expression and localization of the eight mutants in 293T transfected cells. Mutant plasmids 1 through 8 were subsequently used to immunize eight groups of mice through in vivo electroporation (EP). After two EP immunizations (3 weeks apart), we found that a single amino acid mutation, R441A, displayed the same phenotype as the deletion SΔ(422-463) mutant. Similar to SΔ(422-463) immunizations, we failed to induce detectable NAbs (Fig. 3), although binding antibodies were generated (Fig. 4). No NAbs were detected in sera dilutions beginning at 1:10. Apparently, the loss of the major neutralizing domain [in R441A or SΔ(422-463) mutants] resulted in the reduced level of binding antibodies (Fig. 4). Using an indirect ELISA, we further quantified the level of binding antibodies generated. We found that R441A or SΔ(422-463) mutant was able to induce an antibody response mainly against protein S400, with a weak response to S600, at a dilution of 1:50. Conversely, the R453A mutant and OPT9 induced a high level of binding antibody against protein S600 (1:4,050) but not S400 (<1:50). One possible explanation is that the R441A or SΔ(422-463) mutant likely resulted in an altered conformational structure of the RBD, which may deter the induction of neutralizing antibodies. Moreover, ELISpot analysis revealed a relevant cytotoxic T-lymphocyte response to the R441A mu-

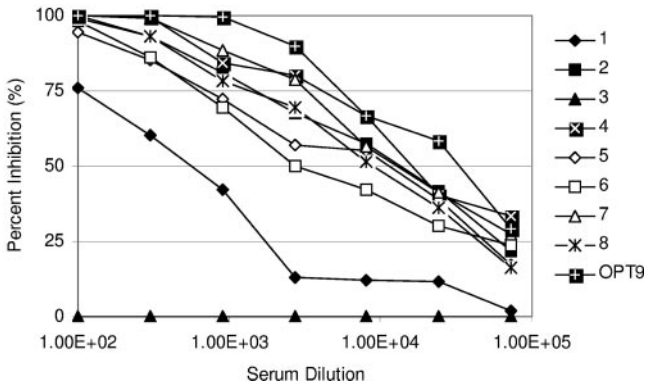

FIG. 3. SARS-CoV-specific neutralization antibody response in BALB/c mice after EP DNA vaccinations. Each group (2 mice per group) was immunized with 20 μg of mutants (1 to 8) or OPT9 plasmid twice, 3 weeks apart. Serum was collected and diluted from 1:100 to 1:72,900 and measured for the ability to neutralize OPT9 pseudovirus. The average values are plotted.

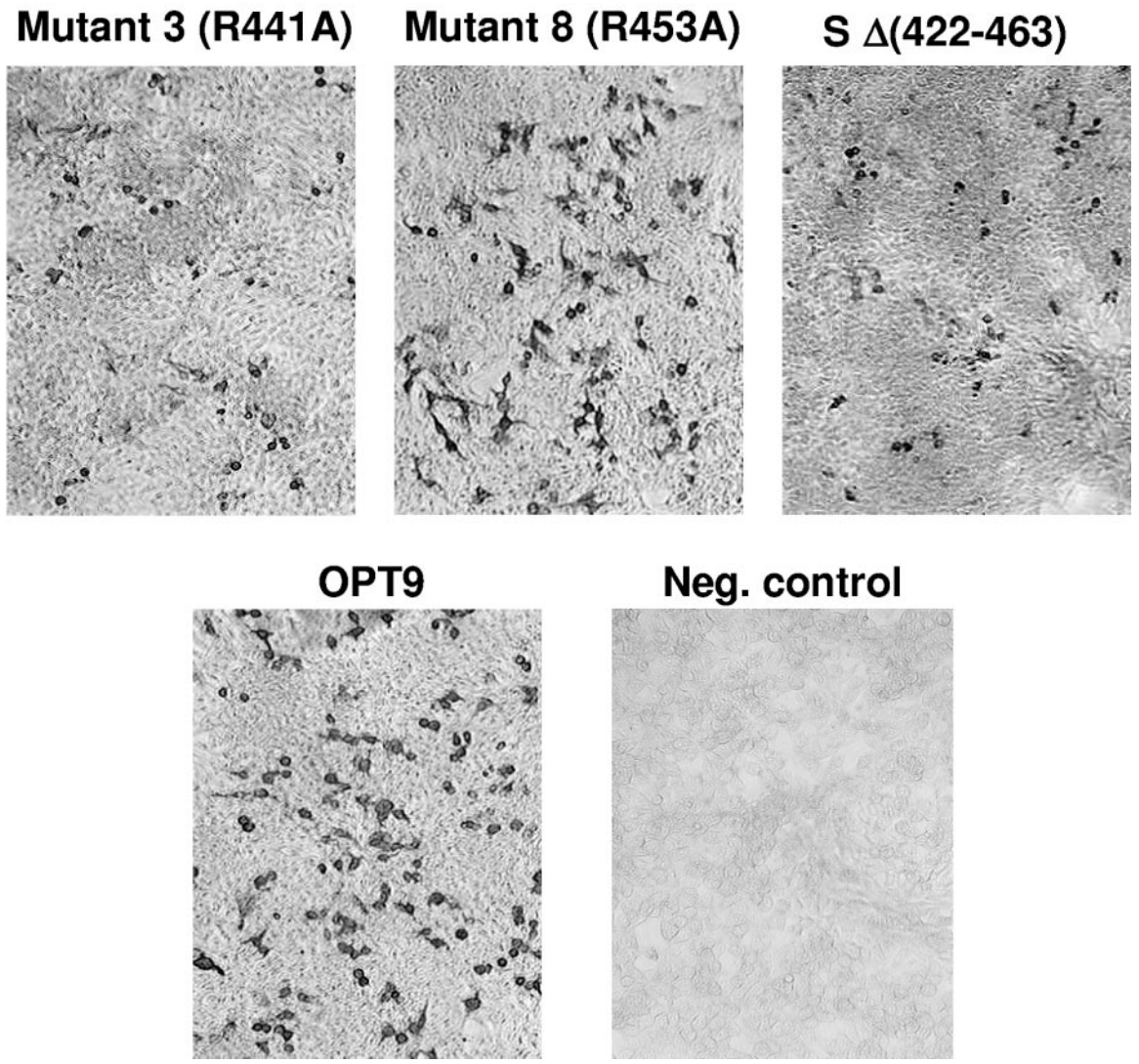

FIG. 4. Detection of binding antibodies to S glycoprotein in vaccinated mice. Sera (1:50 dilution) of mice vaccinated with OPT9, mutant 3, 8, or SΔ(422-463) were used to stain 293T cells transiently transfected with OPT9 plasmid. Binding antibodies were present in each vaccinated mouse. Neg., negative.

tant-immunized mice when compared with pcDNA3.1-OPT9-vaccinated mice (data not shown), providing further evidence that the R441A mutant glycoprotein remained immunogenic in vivo. In contrast, the remaining seven mutant plasmids were able to induce NAbs, despite a lower level of NAb response when compared to pcDNA3.1-OPT9-immunized mice (Fig. 3). Interestingly, mutant 1 (R426A) showed a significant reduction in the level of NAb induced (~10-fold less than pcDNA3.1-OPT9-immunized mice). These results indicate that while most positively charged residues in the region contribute to the induction of NAbs, the R441 residue is indispensable or unchangeable for the immunogenicity of a major NAb domain. Of note, two additional mice were subsequently vaccinated with the R441A mutant, using the same immunization procedure. Again, no detectable NAbs were induced, although binding antibodies were detected. To preclude the possibility of host factor immune interference, we boosted two of four mice that were preimmunized with mutant 3 (R441A) with pcDNA3.1-OPT9. High levels of NAbs to OPT9 pseudovirus were induced after two immunizations, as shown in Fig. 5. Therefore, the data indicate that the basic residue R441 is critical for inducing NAbs.

Positively charged residues within the deletion region of S glycoprotein determine viral entry into target cells. To determine the basic residues necessary for mediating viral entry, we examined the functionality of each of the eight mutants when pseudotyped into virus. We generated eight pseudoviruses using each of the mutant S genes (mutants 1 to 8) in parallel with pcDNA3.1-OPT9. We were able to detect similar levels of mutant S protein in the viral lysates by immunoblotting with a rabbit R419D57 serum (data not shown). The pseudoviruses generated were subsequently used to infect HEK293T-ACE2 cells with an equal amount of p24 input (10 ng). This experiment was repeated three times with three to five replicates each time. All of the mutants, except for mutant 2 (R444A), consistently displayed diminished entry efficiencies by 25 to

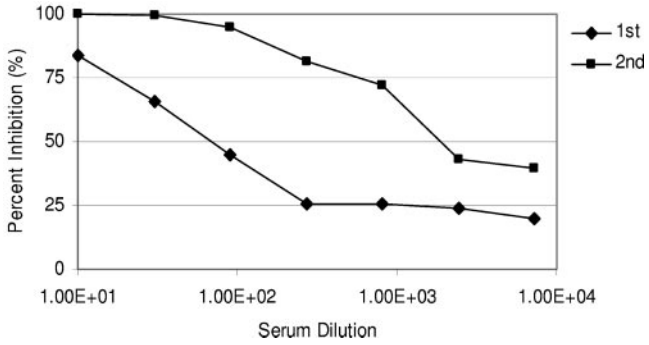

FIG. 5. Induction of SARS-CoV neutralizing antibodies with OPT9 in mice prevaccinated with mutant 3 (R441A). Nonresponsive mice immunized with mutant 3 were subsequently immunized with two EP injections of OPT9, 3 weeks apart. Serum was collected after each injection (1st, 2nd) and analyzed for the ability to neutralize OPT9 pseudovirus at dilutions of 1:10 to 1:7,290. The average NAb titer of two mice is presented.

100% (Fig. 6). Strikingly, mutants 3 (R441A) and 8 (R453A) completely abolished the entry capability of the corresponding pseudoviruses (Fig. 6). We conducted parallel studies with HEK-293T cells and found that none of the pseudoviruses infected these cells (data not shown). These results suggest that residues R441 and R453 are essential for ACE2-mediated viral entry. In particular, R441 contributes to both immunogenicity and receptor interaction. Of note, basic residues R441 and R453 are conserved in all published sequences of human-derived SARS-CoV and civet-derived SARS-CoV-like strains. A recent study identified L-SIGN as an alternative receptor for SARS-CoV (19). In parallel with HEK293T-ACE2 cell infection, we determined whether the panel of our pseudoviruses would change their efficiency for entering L-SIGN-positive cells. Unexpectedly, the cell line NIH 3T3/MX-L-SIGN did not support the infection of any of these pseudoviruses. To avoid host restriction of HIV-1 replication in mouse 3T3 cells, we generated a similar pseudovirus using mouse leukemia virus as a backbone, which contains a green fluorescent protein (GFP) reporter gene. After repeating the test with the GFP pseudoviruses, we did not observe any indication of viral entry (data not shown), although the majority of HEK293T-ACE2 cells expressed GFP. Therefore, we were unable to determine the entry efficiency of our mutant pseudoviruses mediated by the alternative receptor L-SIGN (19). This finding likely suggests that L-SIGN may require an additional cofactor(s) in NIH 3T3 cells to become a functional receptor.

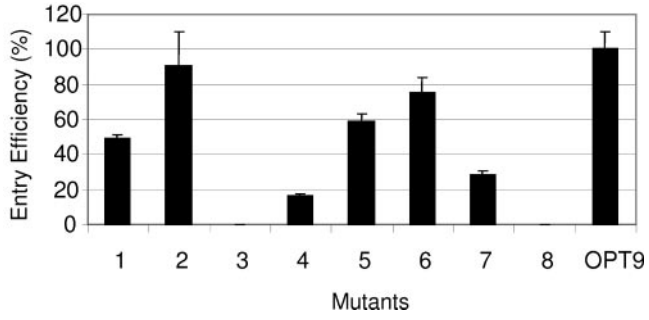

FIG. 6. Functional analysis of mutant pseudoviruses. Ten nanograms (measured by p24) of each pseudovirus was used to infect HEK293T-ACE2 cells. Luciferase activity was measured 72 h postinfection and normalized with OPT9 infection (100%). Five replicates were tested in each experiment. The average values and standard errors are presented. This experiment was repeated three times.

Susceptibility of mutant pseudoviruses to NAbs. In light of a recent publication by Yang et al. (37), which describes antibody-enhanced infectivity of civet-derived SARS pseudoviruses, we tested whether any of our pseudotype mutant viruses were susceptible to neutralization or enhancement with various immune sera. We obtained sera from two immunized mice and two convalescent SARS patients. From the two immunized mice sera, one was derived from two DNA (pcDNA3.1-OPT9, DD131) immunizations and the other was from two ADS-MVA (MM3) vaccinations (6). The two human sera were collected from patients numbered 14 and 48, who were infected by the circulating SARS-CoV strain in 2002. We tested these sera for the ability to neutralize each of our functional mutant viruses (mutants 1, 2, 4, 5, 6, and 7; Fig. 6) and compared this with the neutralizing profile of our wild-type pseudotype virus (OPT9). All of the viruses were susceptible to neutralization when tested by each of the four sera in HEK293T-ACE2 cells (Fig. 7). We did not detect any antibody-mediated enhancement of infection. We repeated this experiment with 786-O cells to determine whether the antibody-mediated phenomenon is dependent on cell type. Unexpectedly, both our pseudotype mutant and wild-type viruses failed to infect 786-O cells with or without immune sera (data not shown). Similar results were observed when 786-O cells were tested with pseudoviruses expressing GFP instead of luciferase (data not shown). Therefore, the mutations we introduced into the deletion region are unlikely involved in the antibody-mediated enhancement, yet the cell type and viral dependence issues remain to be determined.

DISCUSSION

In this study, we removed a positively charged region SΔ(422-463) on the surface of the SARS-CoV S glycoprotein, abolishing its capability to induce neutralizing antibodies and to mediate viral entry into ACE2 cells. These findings highlight the essential role of this region in determining viral tropism and the immunogenicity of a major dominant neutralizing epitope. Our data provide direct functional evaluation of the mutant S glycoproteins by using EP in vivo DNA immunization to enhance immunogenicity, along with a pseudotype-based assay to determine viral entry. We show that the positively charged residues of the deletion region are likely essential for the observed phenotypes, as determined by mutagenesis. In particular, the single amino acid substitution R441A abolishes both immunogenicity and viral entry, whereas another single mutation, R453A, only blocks viral entry. Our findings have critical implications for SARS vaccine and antiviral design as well as for understanding the interaction between the SARS-CoV envelope and the ACE2 viral receptor.

The newly identified deletion region accounts for one of the major immunodominant domains for inducing NAbs. Since the S glycoprotein is the key protein on the viral surface involved with receptor interaction and immune recognition, it serves as the central target for SARS vaccine design. Defining the neu-

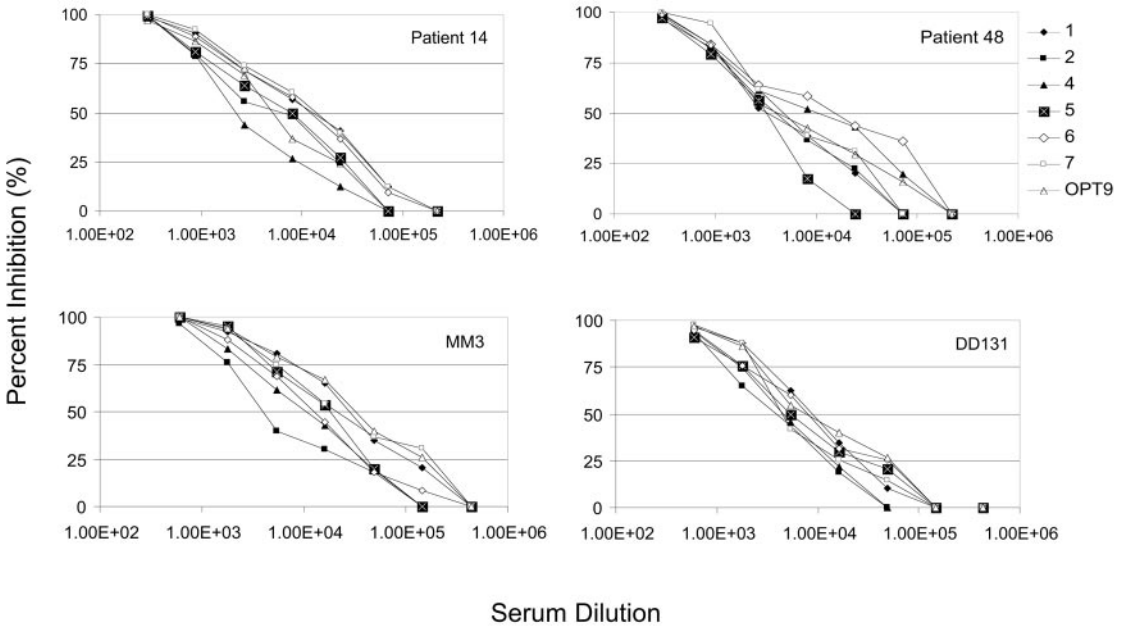

FIG. 7. Susceptibility of mutant pseudoviruses to neutralization with sera derived from immunized mice and human convalescent-phase sera. BALB/c mice vaccinated twice with OPT9 (DD131) or ADS-MVA (MM3) were analyzed for the ability to neutralize the functional mutants (1, 2, 4, 5, 6, and 7) and OPT9 pseudoviruses. Serum dilutions ranged from 1:600 to 1:48,600. Human convalescent-phase sera from patients exposed to SARS-CoV (patients numbered 14 and 48) were analyzed for the ability to neutralize the functional mutants (1, 2, 4, 5, 6, and 7) and OPT9 pseudoviruses. Serum dilutions ranged from 1:300 to 1:24,300.

tralizing domain within the S glycoprotein has been a major emphasis for vaccine development. Antigenic epitopes were previously mapped to S(603-634), which reacted with all the convalescent-phase sera from 42 SARS patients (17). Moreover, patient convalescent-phase sera with high neutralizing activity recognized 11 peptides [S(49-62), S(295-306), S(323-334), S(467-480), S(545-558), S(553-564), S(651-662), S(663-674), S(695-708), S(737-748), and S(879-890)] of the S glycoprotein, suggesting that antibodies against the epitopes represented by these peptides could be responsible for much of the SARS-CoV neutralizing activity (14). Since there is no evidence that these antigenic peptides can currently induce NAbs in vivo, these peptides may not be neutralizing determinants. Two neutralizing determinants were found in the S2 portion [S(803-828) and S(1055-1192)] near the C terminus of the S glycoprotein (21, 40), which overlaps with another identified neutralizing domain [S(797-1192)] (32). Furthermore, neutralizing epitopes were identified in a well-exposed region of S glycoprotein [S(485-625)], mapped to two small regions [S(548-567) and S(607-627)] (42). The S(485-625) region significantly overlaps with S(528-635), a previously defined immunodominant neutralizing epitope (17). In our deleted DNA SΔ(422-463) vaccine, these previously identified antigenic or neutralizing determinants were unchanged, yet we failed to detect the induction of NAbs even though binding antibodies were readily induced. Our data suggest that the deletion region is an indispensable component for inducing NAbs in this major immunodominant domain. Our data also indicate that the previously described neutralizing determinants in S2 may not be properly exposed for inducing NAbs when the plasmid carrying the SΔ(422-463) deletion is used as a vaccine in the context of in vivo EP. Alternatively, these neutralizing determinants likely carry weak and minor epitopes in comparison with the major immunodominant neutralization domain. Our findings, however, do not exclude the possibility that the deletion region may form the major immunodominant neutralization domain in conjunction with other regions. Considering the fact that convalescent-phase sera do not readily react with linear peptides derived from the deletion region (14), we speculate that the major immunodominant neutralization domain is likely conformational. This speculation is supported by recent findings that most potent monoclonal neutralizing antibodies recognize conformational structures of RBD in the S1 glycoprotein (13, 18). Further characterization of these conformational structures is, therefore, highly desirable. Next, we showed that single positively charged residues in the deletion region determine the immunogenicity of the major neutralizing domain. Previous studies indicate that a single amino acid substitution may have a great impact on the biological properties of coronaviruses. For example, a single point mutation (A528V) of bovine coronavirus S glycoprotein was responsible for the escape of MAb binding of the mutant protein (38). Moreover, a single amino acid substitution (A219S) in the S glycoprotein may alter the in vivo tropism of transmissible gastroenteritis coronavirus (1). In relation to SARS-CoV, it was recently reported that the binding capacity of one of the most potent human neutralizing MAbs was reduced with a single mutation of N479S (31). Here, we found that the single positively charged amino acid R441 in the S glycoprotein is indispensable for determining immunogenicity of a SARS-CoV major neutralization domain. After mutating this basic residue to alanine, we showed that the R441A mutant DNA was unable to

induce NAbs in mice, although binding antibodies were induced. Interestingly, R441A mutant-induced binding antibodies tend to react mainly to protein S400, whereas OPT9-induced antibodies primarily react to RBD of S600. These results suggest that it is possible the B-cell response has been shifted to other non-NAb epitopes following the R441A substitution. This phenomenon has been previously described for another immunogen after a single amino acid change (R68E) (8). In addition to the R441A mutation, R426A (mutant 1) substitution also had a profound reduction on the level of NAb (Fig. 3). It is of interest to determine why these two basic residues are critical for the immune recognition of the major NAb B-cell domain, whereas other mutants had minimal effects. Further studies will be required to address this issue. It is possible that other mutations may disrupt the RBD conformation and cause similar effects. Our findings do not exclude the possibility that NAb can be induced against other regions or mutations of S protein when the vaccine is constructed and delivered differently, e.g., as a recombinant protein form or using viral vector systems.

The electrostatic property of the SΔ(422-463) region in the receptor binding domain of the S glycoprotein is critical for viral entry via the cellular receptor ACE2. The interaction between the NAb domain of the S glycoprotein and the cellular receptor ACE2 was initially evident by identifying a potent human neutralizing MAb that binds to the S1 protein and blocks receptor association (30). We previously demonstrated that vaccine-induced NAbs neutralize SARS-CoV through blocking the interaction between virus and the cellular receptor ACE2 (6). Consistent with these findings, the receptor binding domain [S(318-510)] alone was recently found to be sufficient for inducing high levels of NAbs (15). With more MAbs being identified, the binding site of one of the most potent neutralizing MAbs was also mapped to the receptor binding region (31). In addition, SARS convalescent-phase sera are frequently found to specifically compete off binding of these neutralizing MAbs to whole SARS-CoV or the receptor binding domain (RBD) (2), suggesting the in vivo relevance of the major neutralizing epitope. In this study, we showed that most single R to A, K to A, and H to A substitutions, except for an R426A mutant, significantly reduced viral entry efficiency by 25 to 100% (Fig. 6). In particular, R441A and R453A mutants completely abolished its capability to enter the target HEK293T-ACE2 cells. Considering the surface of ACE2 is highly negatively charged (27), we speculate that the electrostatic interaction between viral envelop and receptor is essential for mediating viral entry. In support of this notion, it was previously shown that a single amino acid mutation, D454A, abolished association of the full S1 domain with ACE2 (34). Since R453 and D454 are adjacent to each other, we believe that this region can potentially serve as a major target for designing antiviral agents to block viral entry. Importantly, by introducing an R453A mutation while retaining R441 in the S glycoprotein, it is now possible to make a safer vaccine through blocking ACE2-mediated cell fusion or entry without disturbing the immunogenicity of the major B-cell NAb domain. Finally, we determined that the entry of our mutant pseudoviruses into HEK-293T-ACE2 cells is not enhanced by NAbs derived either from vaccinated animals or human convalescent-phase sera. As another member of the coronavirus family, feline infectious peritonitis virus was shown to contain the antigenic sites in its S glycoprotein responsible for eliciting both neutralization and antibody-dependent infection enhancement (9). It was recently reported that a preexisting antibody response may enhance hepatitis in ferrets experimentally infected with SARS-CoV (33). Since ferrets are the only animal species reported that develop hepatitis after SARS-CoV infection, it remains unclear whether the enhanced hepatitis is merely a ferret-specific phenomenon. Although similar enhanced infection has not been found in other animals, it is disturbing to know that SARS-CoV-specific NAbs may enhance the infection of civet-derived CoVs to enter target 786-O cells in vitro (37). If this is the case in vivo, many current SARS vaccines when applied to humans will potentially cause harm by facilitating cross-species transmission of civet viruses into humans. Considering that the five amino acid differences between SARS-CoV and civet strains were also mapped to the receptor binding region (37), we sought to determine whether the mutations we introduced into the major neutralization domain would also affect the susceptibility of mutant pseudoviruses to NAbs. Using HEK293T-ACE2 as target cells, we found that all of our mutant pseudoviruses tested remain highly sensitive to neutralization, regardless of whether the sera was derived from vaccinated animals or convalescent humans. Therefore, we speculate that these positively charged residues are unlikely involved in the enhancement of infection. Since the newly characterized deletion region is highly conserved among SARS-CoV and related civet strains, we believed that it would be ideal to develop a vaccine containing the major NAb domain while removing the elements for infective enhancement.

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