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NOTES

Identification of Two Neutralizing Regions on the Severe Acute Respiratory Syndrome Coronavirus Spike Glycoprotein Produced from the Mammalian Expression System

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The Spike (S) protein of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) plays important roles in viral pathogenesis and potentially in the development of an effective vaccine against this virulent infectious disease. In this study, the codon-optimized S gene of SARS-CoV was synthesized to construct DNA vaccine plasmids expressing either the full-length or segments of the S protein. High titer S-specific immunoglobulin G antibody responses were elicited in rabbits immunized with DNA against various segments of the S protein. Two neutralizing domains were identified on the S protein, one at the N terminus (Ser12-Thr535) and the other near the C terminus (Arg797-Ile1192).

Severe acute respiratory syndrome (SARS) is a virulent emerging infectious disease and its etiological agent is a new human coronavirus (CoV), known as SARS-CoV (8, 13, 14, 16). Developing an effective vaccine is the most cost-effective approach to achieve protection in a large susceptible population (17). The spike (S) protein of SARS-CoV is a candidate antigen for vaccine development (25). It is a large type-I transmembrane glycoprotein containing 23 potential N glycosylation sites (Fig. 1A) (7, 26) and is responsible for receptor binding and membrane fusion during virus entry (4, 10, 11, 21, 28). The angiotensin-converting enzyme 2 (ACE2) receptor was identified as a receptor for SARS-CoV (10), and a small 193-amino-acid fragment of the S protein (Asn318-Val510) was shown to bind efficiently to ACE2 (24). By using Spike monoclonal antibody (MAb) 80R, isolated from human antibody libraries, a neutralizing region was mapped to Thr261-Lys672 (19). Additional MAbs, generated by *Escherichia coli* over-expressed S fragments, mapped neutralizing epitope to Scr607-Asn627 (28). A different neutralizing epitope Leu803-Ala828 was identified with a peptide which had strong binding to convalescent sera from SARS patients (27). Therefore, it is important to reconcile such differences by using sequential S segments expressed by mammalian systems to map the neutralizing domains on S protein.

In this study, DNA immunization was used as an effective approach to dissect the S neutralizing domains. The codon-optimized S gene derived from the published sequences (13, 16) was chemically synthesized (Geneart, Regensburg, Germany). DNA vaccine plasmids were subsequently constructed to express the full-length and different segments of the S proteins (Fig. 1A). There has been no direct experimental evidence to date suggesting that SARS-CoV S protein is cleaved into S1 and S2 subunits. The designation of S1 (Ser12-Ser798) and S2 (Arg797-Thr1255) was based on the alignment of the SARS-CoV S protein sequence with those of other coronaviruses with known cleavage between their S1 and S2 domains (1, 3, 5, 7, 9, 13, 16). Molecular modeling has proposed a hypothetic division of these two subunits between Leu681-Asp727 (Fig. 1A) (18). S1 was further divided into S1.1 (Ser12-Thr535) and S1.2 (Gly534-Ser798). S2 contains the most conserved regions, such as the heptad repeat (4, 11, 21, 28), transmembrane (TM), and cytoplasmic tail. The DNA plasmid with a deletion of TM, S2.dTM, was also constructed to study the roles of TM in the S posttranslational processing. The S natural leader was replaced by the human tissue plasminogen activator (tPA) leader, which was highly effective in expressing secreted proteins in mammalian cells (2, 15, 22).

All of the codon-optimized S DNA vaccines were highly immunogenic in inducing anti-S immunoglobulin G (IgG) in immunized New Zealand White rabbits (Fig. 1B and C). It recognized both glycosylated and deglycosylated forms of S proteins, either transiently expressed from the 293T cells or associated with SARS-CoV-infected VeroE6 cells (Fig. 1B). Deglycosylation was achieved by using PNGaseF (12, 20). The molecular weight shifts after deglycosylation for every S segment in the analysis (Fig. 1B) supported the notion that the SARS-CoV S protein was glycosylated throughout its entire length (7, 26).

DNA vaccines expressing the full-length S and the N-terminal S1.1 segment elicited high titers, while the S1.2 construct elicited the lowest titer antibodies in recognizing the full-length S antigen (Fig. 1C, left). However, the anti-S1.2 titer became higher when the autologous S1.2 antigen was used as the enzyme-

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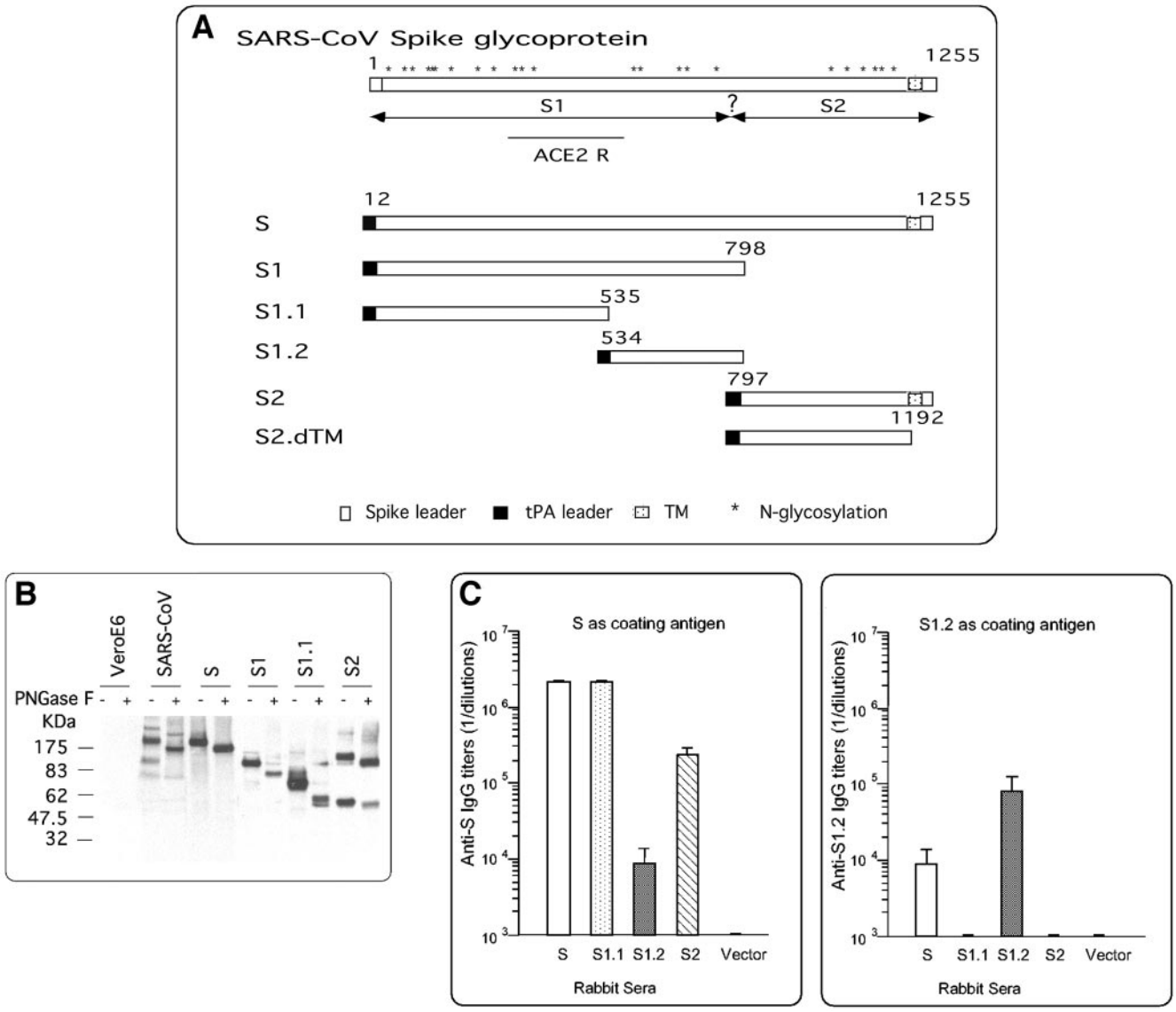

FIG. 1. (A) Designs of SARS-CoV S DNA vaccines. Schematic representation of the entire S protein is shown at the top, including its natural leader and a TM close to the C-terminal tail. Hypothesized N glycosylation sites are marked by asterisks, and the ACE2 receptor (R) binding domain is also noted. DNA vaccines expressing different segments of the S protein are shown in the lower part of the figure, and their amino acid residue numbers are indicated. The nucleotide boundaries for these constructs are 34 to 3,765 bp for S, 34 to 2,394 bp for S1, 34 to 1,605 bp for S1.1, 1,599 to 2,394 bp for S1.2, 2,389 to 3,768 bp for S2, and 2,389 to 3,576 bp for S2.dTM. The tPA leader sequence has replaced the S natural leader in these constructs, and the variant S2.dTM construct has a C-terminal truncation including the TM domain. These codon-optimized S gene segments were individually subcloned into the DNA vaccine vector pSW3891 (22). (B) Western blot with either viron (Urbani strain)-associated S protein (SARS-CoV) or various S segments (S, S1, S1.1, and S2) expressed from transiently transfected 293T cells. Uninfected VeroE6 cells were used as negative controls. Samples were either treated (+) with the PNGaseF or not treated (−), as labeled. Rabbit serum immunized with the full-length S DNA vaccine was used to detect the SARS-CoV proteins. (C) Titers of rabbit anti-S IgG responses after four DNA immunizations as measured by ELISA. Rabbits were immunized with a Helios gene gun (Bio-Rad) on the shaved abdominal skin as previously reported (23). Plasmid DNA (36 μg) was administrated to individual rabbits for each of the immunizations at weeks 0, 2, 4, and 8. Serum samples tested were taken at week 10. Two different S protein antigens, S (left panel) and S1.2 (right panel), expressed from the transiently transfected 293T cells were used as the ELISA coating antigens. Data represent the geometric mean titers of sera from two different rabbits within each group.

linked immunosorbent assay (ELISA) coating antigen (Fig. 1C, right). This indicates that the S1.2 antigen itself was immunogenic but might have poor surface accessibility in the full-length S protein. In addition, the anti-S sera induced by the full-length S protein were less effective in recognizing the S1.2 antigen than that of anti-S1.2 sera (Fig. 1C, right), implying that a large portion of the anti-S IgG targeted non-S1.2 regions.

The specificity of sera induced by segments of the S protein was further confirmed by Western blot analysis using recombinant S proteins from 293T cells. Rabbit anti-S sera recognized the autologous full-length S protein and each of the S segments (Fig. 2A). The anti-S1.1 sera recognized the autologous S1.1 antigen as well as the full-length S antigen and the S1 antigen, which contained the S1.1, but did not recognize the

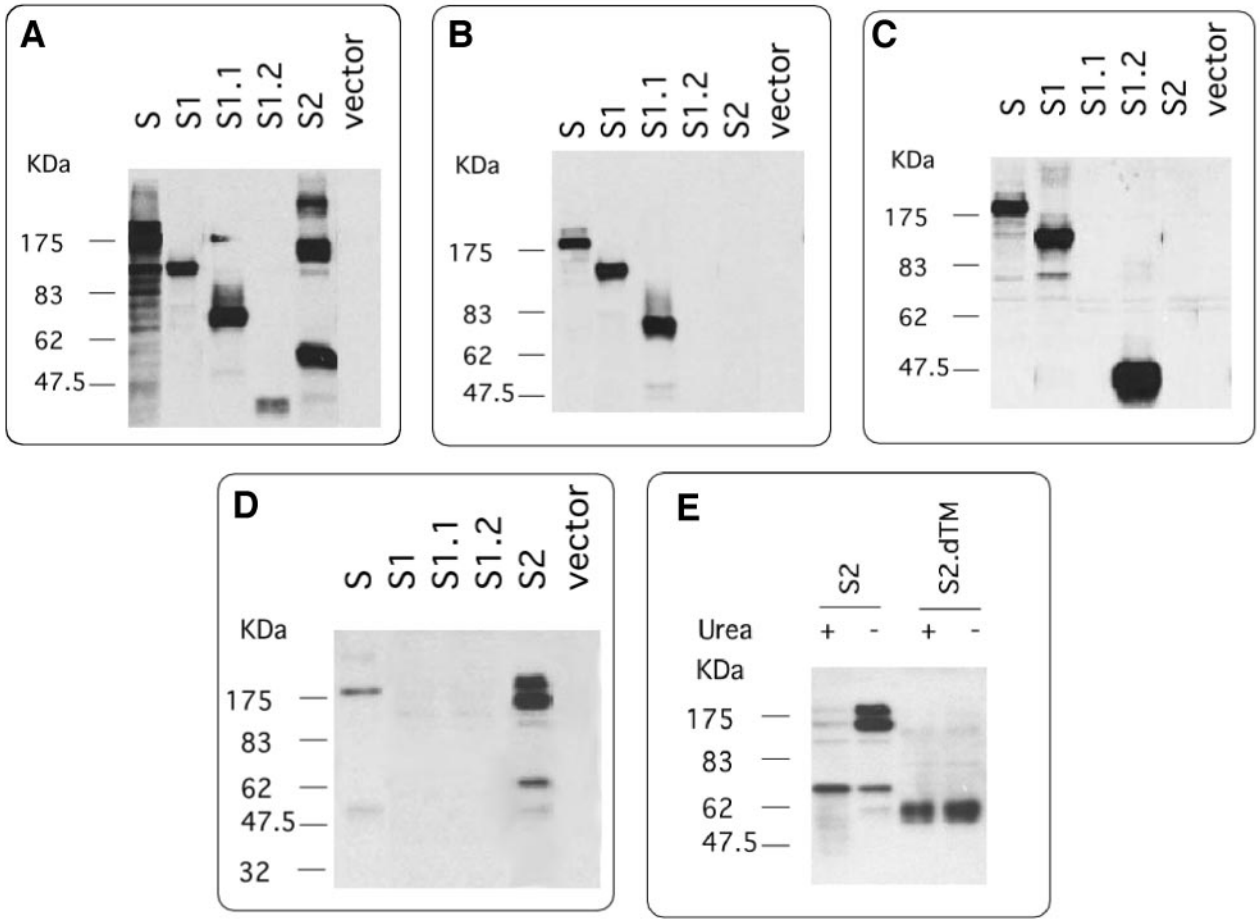

FIG. 2. Western blot analysis to identify the specificity of anti-S rabbit sera elicited by DNA vaccines expressing different S segments. (A) Rabbit serum raised by S DNA vaccine; (B) rabbit serum raised by S1.1 DNA vaccine; (C) rabbit serum raised by S1.2 DNA vaccine; (D) rabbit serum raised by S2 DNA vaccine. Cell lysates from 293T cells transfected with various S-expressing DNA vaccines and the empty vector are labeled above each Western blot. All samples were run under denatured conditions. (E) Western blot analysis to compare the potential of S2 and S2.dTM to form oligomers with rabbit serum immunized with the S2-expressing DNA vaccine. The 293T cell lysates transfected with either of the two S2 DNA constructs were heat (95°C) treated for 5 min prior to their loading onto sodium dodecyl sulfate-polyacrylamide gels; 4M urea was added to half of the samples (lanes labeled +).

S1.2 or S2 antigen (Fig. 2B). Similarly, the anti-S1.2 serum recognized the S1.2 and the two larger S antigens (full-length S and S1), but not the non-overlapping S1.1 or S2 antigen (Fig. 2C). Finally, the anti-S2 serum recognized the S2 antigen and to a lesser degree the full-length S antigen but not any of the other unrelated S segments (Fig. 2D). Three bands were observed in S2 samples (Fig. 2A and D), most likely representing the monomer, trimer, and an even larger oligomer, based on their apparent molecular weights. The potential of S2 to form heat-resistant oligomers was confirmed by an additional experiment in which 4M urea was able to resolve the oligomeric structures (Fig. 2E). The TM region was important for oli-

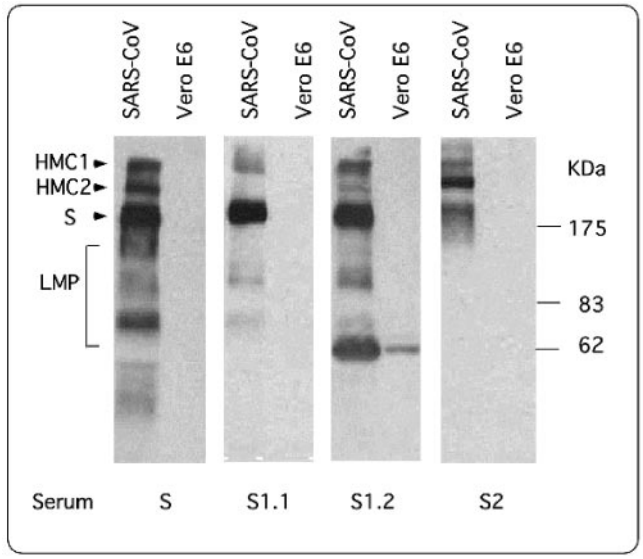

FIG. 3. Western blot analysis of SARS-CoV-associated S proteins by using rabbit sera elicited by DNA vaccines expressing different S segments (S, S1.1, S1.2, and S2). Cell-associated viral samples were collected 4 days postinfection and 100 ng of protein was loaded into each lane for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: SARS-CoV, VeroE6 lysate infected with SARS-CoV; VeroE6, uninfected VeroE6 cells. S, expected monomeric S glycoprotein.

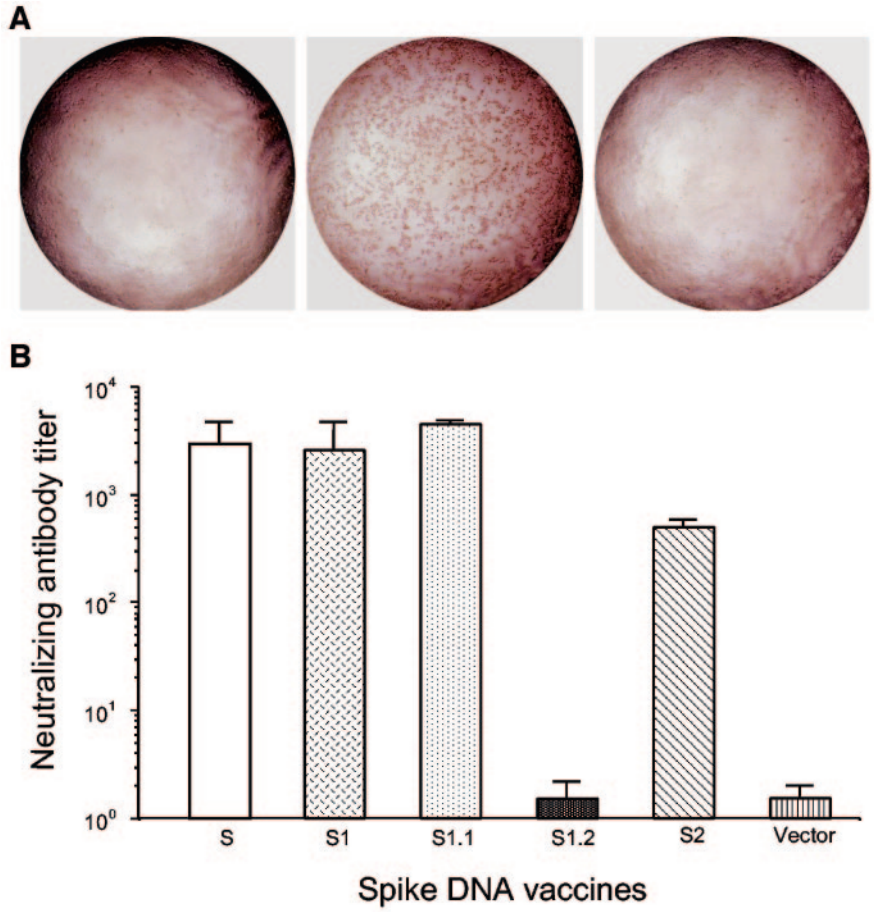

FIG. 4. CPE assay to examine the neutralizing antibody responses in DNA-immunized rabbit sera. The neutralization assays were performed with triplicate testing wells in a 96-well flat-bottom plate in the biosafety level 3 laboratory. SARS-CoV (400 50% tissue culture infective doses) in 50 μl/well was incubated with 50 μl of serially diluted rabbit sera or tissue culture medium for 1 h. After the incubation, 100 μl of VeroE6 cells (20,000 cells) was added to each well at a multiplicity of infection of 0.02. The assay results were determined by CPE assay on day 4 of infection. (A) Sample pictures of uninfected (left) and SARS-CoV-infected VeroE6 cells in the presence (right) or absence (middle) of neutralizing rabbit serum elicited by DNA vaccine expressing full-length S antigen. (B) Anti-S neutralizing antibody titers of rabbit sera elicited by different S DNA vaccines. The neutralizing antibody titers are presented as the geometric means of two rabbit sera at the highest antibody dilutions that could completely prevent CPE.

gomer formations because truncation of the TM would leave the S2.dTM protein in monomer form (Fig. 2E).

All four rabbit sera, raised either by the full-length S or segments of the S protein, recognized a dominant band with a size of approximately 175 kDa in SARS-CoV-infected VeroE6 lysates, as expected from a monomeric S protein (Fig. 3). Our data demonstrated the possible proteocleavage on the S protein leading to several smaller low-molecular-weight products (LMPs), which were detected by anti-S, anti-S1.1, and anti-S1.2 sera but not by anti-S2 sera. Meanwhile, two high-molecular-weight complexes (HMC1 and HMC2) were observed. The HMC2 band was detected mainly by the anti-S and anti-S2 sera but was not effectively detected by the anti-S1.1 and anti-S1.2 sera (Fig. 3). These data support the possibility that the SARS-CoV-associated S was first cleaved to S1 and S2; S1 was further cleaved to generate the LMPs, and S2 stayed as oligomers, which might be stabilized through the C-terminal TM domain similar to that shown in Fig. 2E. The other high-molecular-weight complex, HMC1, was recognized by the anti-S, anti-S1.1, and anti-S1.2 sera and to a lesser extent by the anti-S2 sera, suggesting that HMC1 most likely is an oligomer formed by the full-length S proteins as reported previously (4, 11, 21, 28) and may serve as the proteocleavage precursor for LMPs and HMC2.

Neutralization of SARS-CoV infection to VeroE6 cells was further examined by cytopathic effect (CPE) assay. Sample pictures of uninfected and infected VeroE6 cells are shown in Fig. 4A, and the neutralizing titers based on the CPE assay are summarized in Fig. 4B. The S, S1, and S1.1 DNA vaccines elicited strong neutralizing antibody responses while the S2 vaccine also elicited positive neutralizing antibody responses. S1.2 did not elicit significant neutralizing activity against SARS-CoV, even though there were substantial anti-S1.2 IgG binding antibodies (Fig. 1C, right). Our data suggested that there were at least two neutralizing domains on SARS-CoV S protein, one at the N-terminal S1.1 region and the other at the C-terminal S2 segment.

Our finding of two neutralizing domains in S reconciles recently published findings of a neutralizing domain recognized by MAbs 80R (19) and S26 and S78 (28), all within the N ter-

minus of S, and the identification of another neutralizing determinant (Leu803-Ala828) within S2 (27). The C-terminal neutralizing domain has also been detected in another coronavirus, mouse hepatitis virus (6). It is not unusual to identify more than one neutralizing domain on a viral surface protein. Multiple neutralizing epitopes have been identified on the human immunodeficiency virus type 1 HIV-1 envelop glycoprotein, both at the N-terminal gp120 (the V3 loop) and the C-terminal gp41 (the 2F5 epitope) subunits (29). Our data confirmed that DNA immunization is effective in inducing high-level antibody responses against S glycoprotein as reported previously (25). This study also suggested the possibility that the SARS-CoV-associated Spike protein might undergo a stepwise cleavage to generate S1 and S2 proteins and that cleaved S2 segments would remain as oligomers. Further study is needed to determine if this process will affect the fusion and entry of SARS-CoV into targeted cells and whether such cleavage is cell dependent and tissue specific.

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