DNA AND CELL BIOLOGY
Volume 24, Number 8, 2005

Pp. 503–509

Identification and Antigenic Epitope Mapping of Immunodominant Region Amino Residues 510 to 672 on the Spike Protein of the Severe Acute Respiratory Syndrome Coronavirus

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ABSTRACT

The severe acute respiratory syndrome (SARS) is a newly emerging human infectious disease caused by the severe acute respiratory syndrome coronavirus (SARS-CoV). The spike (S) protein of SARS-CoV is a major virion structural protein. It plays an important role in the interaction with receptors and neutralizing antibodies. In this study, the S1 domain of the spike protein and three truncated fragments were expressed by fusion with GST in a pGEX-6p-1 vector. Western blot results demonstrated that the 510–672 fragment of the S1 domain is a linear epitope dominant region. To map the antigenic epitope of this linear epitope dominant region, a set of 16 partially overlapping fragments spanning the fragment were fused with GST and expressed. Four antigenic epitopes S1C3 (539–559), S1C4 (548–567), S1C7/8 (583–606), and S1C10/11 (607–630) were identified. Immunization of mice with each of the four antigenic epitope-fused proteins revealed that all four proteins could elicit spike protein specific antisera. All of them were able to bind to the surface domain of the whole spike protein expressed by recombinant baculovirus in insect cells. Identification of antigenic epitopes of the spike protein of SARS-CoV may provide the basis for the development of immunity-based prophylactic, therapeutic, and diagnostic clinical techniques for the severe acute respiratory syndrome.

INTRODUCTION

THE SEVERE ACUTE RESPIRATORY SYNDROME coronavirus (SARS-CoV) is a newly emerged virus, and has been identified to be the causative agent of the severe respiratory syndrome (SARS) (Drosten *et al*., 2003; Ksiazek *et al*., 2003; Peiris *et al*., 2003). SARS is a highly infectious and fatal disease. During the epidemic of 2002 to 2003 this disease affected approximately 8500 people worldwide and caused over 800 deaths.

SARS-CoV is an enveloped, positive-sense, ssRNA virus. The genome of SARS-CoV is approximately 29.7 kb in length, with 11 open reading frames. Its genomic organization is similar to that of other coronaviruses (Qin *et al*., 2003). SARS-CoV has four main structural proteins, designated S, M, E, and N. Its gene sequence and amino acid sequence have very low homology with any other known animal coronaviruses (Marra *et al*., 2003; Qin *et al*., 2003; Rota *et al*., 2003). Recently, however, SARS-CoV-like viruses were isolated from Himalayan palm civets (Guan *et al*., 2003), which had not been found in any animal or human before 2003. Phylogenetic analysis indicated that the new SARS-CoV and SARS-CoV-like viruses are not related to the known group 1, 2, or 3 coronaviruses, but rather represent a novel type of coronavirus (Eickmann *et al*., 2003). Therefore, it is proposed that they represent a fourth group within the genus *Coronavirus*, the group 4 coronavirus.

The SARS-CoV spike protein has 1255 amino acids. It is a type 1 transmembrane glycoprotein (Bosch *et al*., 2003). Unlike other coronavirus spike proteins, SARS-CoV spike protein was not cleaved into S1 and S2 domains. But according to the conserved motifs alignment and functional analysis, the SARS-

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TABLE 1. PRIMERS USED IN PCR AMPLIFICATION REACTIONS

Name	*Nucleotide sequence*
S1p1	5′-ATAGGATCCAGTGGTAGTGACCTTGACCG-3′
S1p2	5′-CGCCTCGAGTTTTTGGCTAGTACTACGTAA-3′
SRBDp1	5′-CGCGGATCCAATATTACAAACTTGTGTCC-3′
SRBDp2	5′-TAACTCGAGAACCGTGGCCGGTGCATTTA-3′

The underlined sequences are restriction sites.

CoV spike protein can be divided into two domains, S1 and S2 (Li *et al*., 2003; Spiga *et al*., 2003). The S1 domain of the SARS-CoV spike protein determines tropism to target cells and mediates binding with receptors on cells (Li *et al*., 2003; Wong *et al*., 2004). The S2 domain has a transmembrane sequence and enables the spike protein to dock on the envelope membrane of the virus. Like some other virus spike proteins, SARS-CoV spike protein has two conserved heptad repeat regions that mediate viral introduction via virus-host cell membrane fusion (Gallagher and Buchmeier, 2001; Liu *et al*., 2004; Tripet *et al*., 2004). The spike protein has good antigenicity, and may induce neutralizing antibodies. An inactivated SARS coronavirus vaccine, an attenuated virus vaccine, a recombinant spike protein subunit vaccine, and a spike protein encoding DNA vaccine each have the ability to induce protective neutralization antibodies (Bisht *et al*., 2004; Bukreyev *et al*., 2004; He *et al*., 2004; Takasuka *et al*., 2004; Xiong *et al*., 2004; Yang *et al*., 2004). These features make the SARS-CoV spike protein a suitable candidate for genetic engineering of a subunit vaccine, and suggest it may have diagnostic applications.

Epitope mapping is helpful for vaccine design, and epitope-based vaccines have great application prospects. Linear neutralization epitopes have been identified in other coronaviruses (Daniel *et al*., 1993). Recently, some linear epitopes were identified in the SARS-CoV spike protein (Choy *et al*., 2004; Hua *et al*., 2004; Zhang *et al*., 2004; Zhou *et al*., 2004). In this study, with a prokaryotic expression system, we identified four linear antigenic epitopes. All four of these epitopes are on the exposed domain of the SARS-CoV spike protein, and therefore may be useful in the development of effective vaccines against SARS-CoV.

MATERIALS AND METHODS

Expression of S1 and truncated S1 fragments of the SARS-CoV spike protein

The S1 (12–672 amino acid [aa] of SARS-CoV spike protein) encoding fragment was PCR amplified with a pair of primers, S1p1 and S1p2, by using the plasmid pBLUE-SS as a template (Table 1). The pBLUE-SS plasmid harboring the full-length SARS spike protein gene was previously constructed in our laboratory. The SRBD (318–672 aa of SARS-CoV spike protein) encoding fragment was PCR amplified with another pair of primers (Table 1). For SRBD amplification, the forward primer was SRBDp1 and the reverse primer was SRBDp2.

The N terminal fragment of S1 (S1N) was amplified with the S1p1 and SRBDp2 primers, and the C terminal fragment (S1C) was amplified with the SRBDp1 and S1p2 primers. The PCR products were digested with the restriction enzymes *Bam*HI and *Xho*I, recovered, and then cloned into the pGEX-6p-1 plasmid (Invitrogen, Carlsbad, CA). The recombinant plasmids were identified by restriction enzyme digestion and sequencing. The confirmed recombinant plasmid was transformed into *Escherichia coli* strain BL21. After induction with 0.1 M IPTG at 37°C for 4 h, the recombinant fusion protein GST-S1 was expressed in an inclusion body. The inclusion body was denatured with 8 M urea and refolded in Tris-HCl buffer.

Expression and purification of overlapping short fragments covering the 510–672 aa fragment

A set of 16 partially overlapping short peptides S1C1 to S1C16, covering the fragment 510 to 672 aa. were designed as shown in Figure 2A. For each short peptide, a pair of oligonucleotide strands was synthesized. After annealing the two strands, the double-stranded DNA formed a *Bam*HI and an *Xho*I cohesive terminus at the 5′ and 3′ ends, respectively. The annealed fragment was cloned into the expression vector pGEX-6p-1. The inserts in the recombinant plasmids were sequenced. The confirmed recombinant plasmid was transformed into the *E. coli* strain BL21 and the expressed short peptide with a GST tag was purified by Glutathione Sepharose 4B RediPack Column affinity chromatography according to the manufacturer's

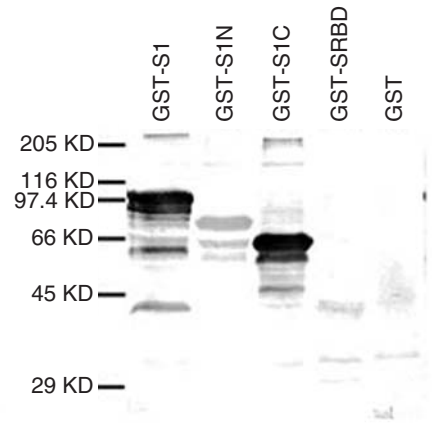

FIG. 1. Western blot analysis of the reactivity of S1 and its truncated fragments with sera from immunized chickens. S1, S1N, S1C, and SRBD were expressed in a fusion protein with GST in the pGEX-6p-1 vector, induced with IPTG and blotted with immunized chicken sera. Molecular standards are shown on the left.

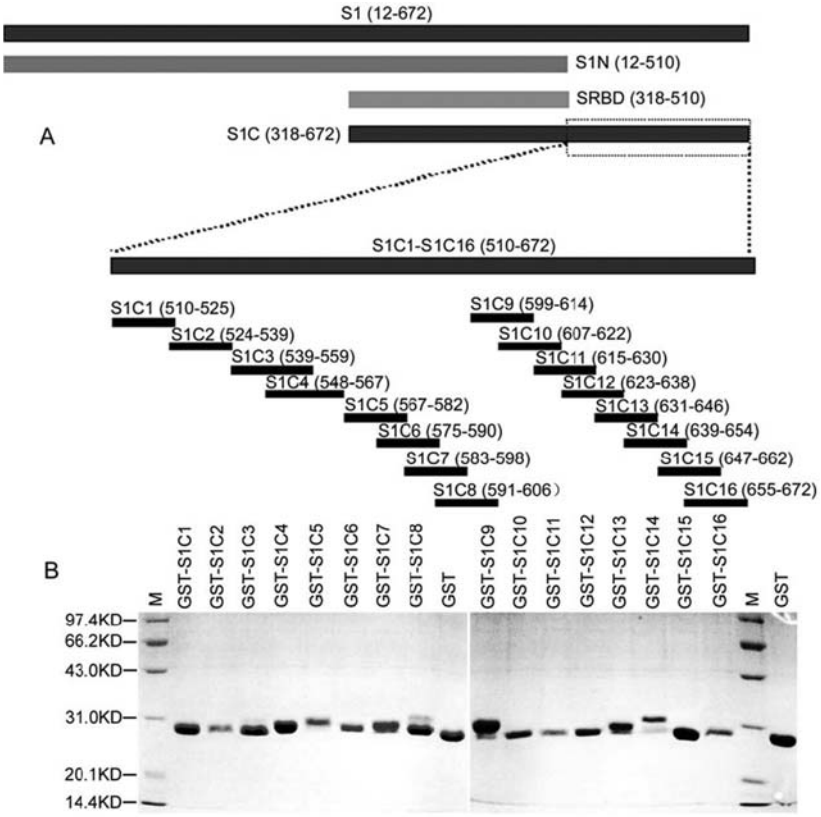

FIG. 2. Expression and purification of fragments of the SARS-CoV spike protein. (**A**) Schematic diagram of relative location of truncated spike protein fragments and overlapping short peptides, S1C1 TO S1C16, spanning the 510–672 aa region. The numbers in parentheses indicate the beginning and the end amino acid of each fragment. S1C1 to S1C16 are a set of partially overlapping short peptides covering the fragments of 510–672 aa of spike protein as the dashed box defined in S1C fragment. Annealed double-stranded DNA fragments were cloned into the expression vector pGEX-6p-1. (**B**) Purification of 16 short peptide-fused recombinant fusion proteins. After being induced with IPTG, the supernatants of sonicates were purified by affinity chromatography. The purified proteins were analyzed by 12% SDS-PAGE and stained with Coomassie brilliant blue. M designates the molecular standards as labeled on the left.

instructions (Amersham Pharmacia Biotech, Arlington Heights, IL). Subsequently, the bound fusion protein was eluted with glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0) for further analysis.

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well microtiter plates were coated with purified fusion protein or bacterial sonicates in 0.1 M carbonate buffer

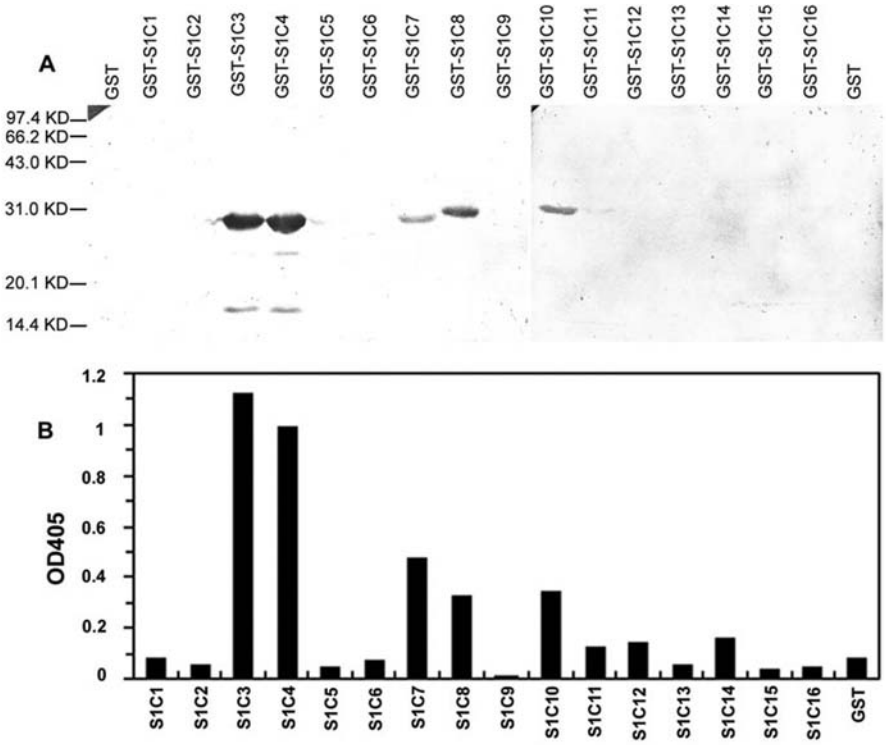

FIG. 3. Identification of the antigenic determinants on the 510–672 aa fragment with sera from immunized chickens. (**A**) Western blot analysis of short peptide-fused proteins revealed that GST-S1C7, GST-S1C8, and GST-S1C10 are reactive with immunized chicken sera. There was strong reactivity of sera with GST-S1C3 and GST-S1C4 and weak reactivity with GST-S1C11. (**B**) ELISA analysis of fusion short peptide. Microtiter plates were coated with purified recombinant fusion protein samples (2 μg/100 μl per well). After blocking with skim milk, chicken sera [1:200] was added, followed by adding a secondary antibody AP-coupled goat antichicken IgG.

(pH 9.6) at 4°C overnight and blocked with 5% skim milk for 3 h. After blocking, the plates were washed three times with PBST (PBS with 0.1% Tween 20). In the binding assay, the plates were incubated with diluted chicken hyperimmune sera (provided by the SARS group of Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences) at 37°C for 1 h followed by washing three times with PBST. Bound antibodies were detected with an alkaline phosphatase (AP)-conjugated rabbit antichicken IgG secondary antibody (Sigma, St. Louis, MO). The reaction was stopped with 2 M NaOH and the absorbance was measured at 405 nm by a microplate auto-reader (Bio-Rad, Hercules, CA). In competitive ELISA, before incubation with coated microtiter plates, the primary antibody was first incubated with each short peptide-fused protein at 37°C for 30 min. In immunized mice sera titering ELISA, microtiter plates were coated with recombinant baculovirus (rBac-SS) expressing the full-length spike protein of SARS-CoV (Wang *et al*., submitted). The primary antibodies were diluted in immunized mice sera. AP-conjugated horse antimouse IgG (Sigma) was used as the secondary antibody.

Western blotting

Cell lysates or proteins were mixed with an equal volume of sample loading buffer (50 mM Tris/HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE). For immunoblotting, proteins were transferred from the SDS-polyacrylamide gel to a nitrocellulose membrane. Nonspecific antibody binding sites were blocked with 5% skim milk in PBS overnight at 4°C. The membranes were incubated with primary antibody at 37°C for 1 h. After incubation with the primary antibody, the membrane was washed three times with PBST (10 min each time). The blot was then probed with the appropriate secondary antibody (1:5,000) for 1 h at 37°C. Secondary antibodies were AP-conjugated goat antimouse IgG (Sigma), HRP-conjugated goat antihuman IgG (Beijing Zhongshang Biotechnology Co., Ltd Beijing, China) or AP-conjugated rabbit antichicken IgG (Sigma). Following secondary antibody incubation, each blot was washed three times with PBST, and then developed with either the HRP or the AP developer solution. For Western blot analysis of the immunized mice sera with full-length spike protein, the cell lyses were separated by SDS-PAGE with 10% polyacrylamide gels.

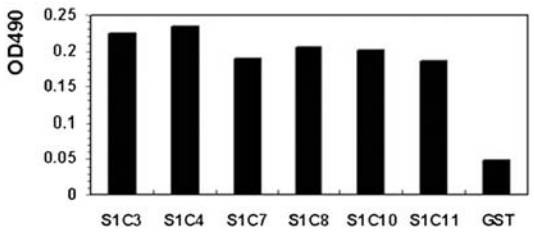

FIG. 4. ELISA analysis of the fused short peptide with SARS convalescent sera. All six fusion proteins could be recognized by SARS convalescent sera.

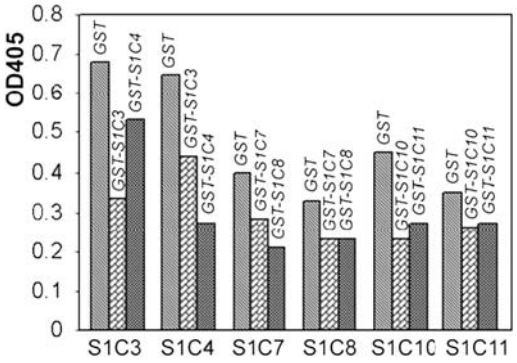

FIG. 5. Competitive inhibition ELISA with overlapping epitopes. The values shown represent the results from a single experiment. The experiment was repeated with similar results.

Immunization experiments

Each of five groups of three 6-week-old BALB/c mice were injected with 50 μg of purified fusion protein: S1C3, S1C4, S1C7, S1C8, or S1C10. For the first immunization, the fusion protein was emulsified with an equal volume of complete Freund's adjuvant (Sigma). Animals were injected at 2-week intervals with the same immunogen mixed with incomplete Freund's adjuvant. One week after the second booster immunization, antiserum samples from immunized animals were collected.

Immunofluorescence assays

Monolayers of Sf9 cells were infected with recombinant baculovirus rBac-SS at a Moi of 2 and were incubated for 72 h at 28°C. The cells were harvested by centrifugation and washed twice in PBS. Glass slides were coated with infected cells, air dried, and fixed with acetone. Immunofluorescence assays were processed with epitope specific mice sera employed as the primary antiserum and a FITC-conjugated goat antimouse IgG (Jackson IR) as the secondary antibody. Samples were analyzed with a Leica microscope, and images were acquired with a Leica digital camera.

RESULTS

The 510–672 aa fragment is the linear epitope dominant region in the S1 domain of the SARS-CoV spike protein

To identify linear antigenic epitope-dominant regions within the S1 domain of the spike protein, S1 (12–672 aa) and three truncated fragments, S1N (12–510 aa), S1C (318–672 aa), and SRBD (318–510 aa) were expressed as a fusion protein with GST in the pGEX-6p-1 vector. The hyperimmune chicken sera recognized all four recombinant fusion proteins in the Western blot assay. Among the four recombinant fusion proteins, S1 and S1C demonstrated the strongest binding with the chicken sera, whereas S1N, and especially SRBD, demonstrated only very weak sera binding (Fig. 1). From these data it is reasonable to deduce that the 510–672 aa fragment is the linear epitope dominant region in S1 of the SARS-CoV spike protein.

Mapping of antigenic epitopes on the 510–672 aa fragment of the SARS-CoV spike protein

To map the antigenic epitope of the 510–672 aa fragment, 16 partially overlapping fragments (S1C1–S1C16) were designed with lengths ranging from 16 to 21 amino acids spanning the 510 to 672 aa fragment (Fig. 2A). All fragments were expressed in fusion with GST in the pGEX-6p-1 vector. The recombinant fusion proteins were purified with Glutathione Sepharose 4B Redi-Pack column affinity chromatography according to the manufacturer's instructions (Amersham-Pharmacia Biotech) (Fig. 2B). ELISA and Western blot assays with immunized chicken sera were carried out for antigenicity analysis of the 16 recombinant fusion proteins. The ELISA (Fig. 3B) and Western blot (Fig. 3A) results similarly revealed six fragments that were recognized by the chicken sera. The ELISA results further demonstrated that they all could be recognized by SARS convalescent sera (Fig. 4). The six sera-binding fragments include three pairs with overlapping sequences. The S1C3 and S1C4 pair shares a 12 aa common sequence, while the S1C7 and S1C8 pair, and the S1C10 and S1C11 pair each shares an 8-aa common sequence. To investigate whether the antigenicity of these peptides is due to the overlapping parts of the sequences or to unique antigenic determinants within each fragment, competitive ELISA were carried out for each pair of overlapping short peptide fused proteins. The results revealed the following four antigenic epitopes of the SARS-CoV S1 domain: S1C3 (539–559), S1C4 (548–567), S1C7/8 (583–606) and S1C10/11 (607–630) (Fig. 5).

Immunogenicity of the four antigenic epitopes

To investigate whether these four epitopes could elicit humoral immune response in mice, five groups of three 6-week-old BALB/c mice were each injected with 50 μg of the purified fusion protein S1C3, S1C4, S1C7, S1C8, or S1C10. One week after the second booster immunization, antiserum samples of immunized animals were collected. In ELISA, all five fusion proteins induced spike protein specific antibodies, and the dilution titers of each exceeded 640. In the Western blot assay, however, only S1C4 and S1C10 induced spike protein specific antibodies (Fig. 6).

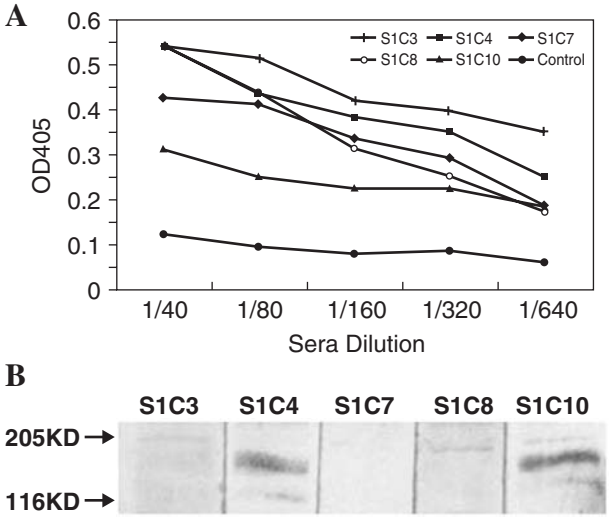

FIG. 6. ELISA and Western blot analysis of epitope-specific mice antisera. (**A**) ELISA analysis of the reactivity of immunized mice sera to recombinant baculovirus expressed full-length spike protein. (**B**) Western blotting analysis of the reactivity of antisera with full-length spike protein. The antisera against S1C4 and S1C10 could recognize the full-length denatured spike protein.

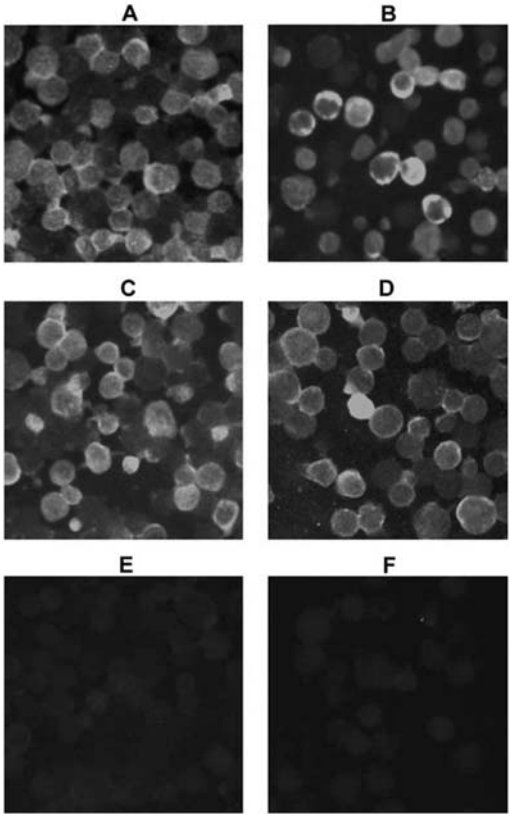

FIG. 7. Immunofluorescence analysis of epitope-specific mice antisera. Immunofluorescence assay of harvested and fixed Sf9 cells infected with full-length spike protein expressing recombinant baculovirus with epitope-specific mice sera as the primary antibody and a FITC-conjugated goat antimouse IgG as the secondary antibody revealed that immunized mice sera against S1C3 (**A**), S1C4 (**B**), S1C8 (**C**), and S1C10 (**D**) all recognized infected Sf9 cells. The control nonimmunized mouse sera did not recognize the same infected Sf9 cells (**E** and **F**).

The four antigenic epitopes are exposed on the surface of the whole spike protein expressed by baculovirus in insect cells

To investigate whether the antisera from the immunized mice could recognize the full-length spike protein expressed by recombinant baculovirus in insect cells, we stained the recombinant baculovirus infected Sf9 cells with these antisera and control nonimmunized mice sera. The antisera against S1C3, S1C4, S1C8, and S1C10 all specifically bound infected Sf9 cells while the nonimmunized mice sera did not (Fig. 7).

DISCUSSION

SARS is a highly infectious and potentially lethal disease. To prevent or control future outbreaks of this disease, the develop-

ment of an effective SARS vaccine is urgently needed. Presently, some data suggest that protective humoral immunity is achievable (Nie *et al*., 2004; Subbarao *et al*., 2004; ter Meulen *et al*., 2004; Zheng *et al*., 2004). Neutralizing antibodies could be induced by inoculation with inactivated vaccines, DNA vaccines, attenuated viruses encoding SARS-CoV spike protein or expressed spike protein (Bisht *et al*., 2004; Bukreyev *et al*., 2004; He *et al*., 2004; Takasuka *et al*., 2004; Xiong *et al*., 2004; Yang *et al*., 2004). For humoral protective immunity, the spike protein of SARS-CoV is the only significant neutralization and protective antigen among the viral structural proteins (Buchholz *et al*., 2004). However, there are serious concerns about safety and efficacy (Marshall and Enserink, 2004). A recent study demonstrated that SARS-CoV infection of ferrets caused mild liver inflammation, and the liver damage became much more serious if the ferrets were first immunized with vaccinia virus-based SARS vaccines before the virus challenge (Weingartl *et al*., 2004).

In this study, we identified four linear antigenic epitopes on the S1 domain of the spike protein of SARS-CoV: S1C3 (539–559), S1C4 (548–567), S1C7/8 (583–606), and S1C10/11 (607–630). Although S1C3 and S1C4 share a common 12 aa sequence, the competitive ELISA results demonstrates that they present two different epitopes. S1C3 only partially inhibited the binding of S1C4 to chicken hyperimmune sera but significantly inhibited its own binding to chicken sera. Likewise, S1C4 weakly inhibited the binding of S1C3 to chicken sera but significantly inhibited its own binding to chicken sera. Among the four identified epitopes, the S1C4 and S1C10/11 epitopes correspond with the results of a prior study in which the two epitopes were identified with monoclonal antibodies. The previous work showed that the 548–567 aa epitope (designated S1C4 here) is a neutralization epitope (Zhou *et al*., 2004). Epitopes S1C3 and S1C7/8 were first reported here. The further characterization of these linear epitopes may provide useful information for understanding the function and structural of spike protein even for vaccine design.

S1C3 and S1C4 were strongly recognized by immunized chicken sera both in Western blot and ELISA analyses, indicating that they are immunodominant epitopes. Presently, data about the host immune response to different epitopes during the course of the disease are lacking. This information will clearly be critical for enabling an effective SARS vaccine to be designed as well as for the development of SARS diagnostics and therapies.

ACKNOWLEDGMENTS

This work was partially supported by a Heilongjiang postdoctoral grant. We greatly appreciate all of the SARS group colleagues at the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences for providing inactivated SARS-CoV sonicates antigen and chicken sera. We thank Dr. Ming Liao and Dr. Guihong Zhang of Southern China Agricultural University for providing SARS convalescent serum.

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Received for publication December 7, 2004; accepted January 7, 2005.