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Research Article

Identification of Immunogenic Determinants of the Spike Protein of SARS-like Coronavirus*

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Bat SARS-like coronavirus (SL-CoV) has a genome organization almost identical to that of SARS-CoV, but the N-terminus of the Spike (S) proteins, which interacts with host receptor and is a major target of neutralizing antibodies against CoVs, of the two viruses has only 63-64% sequence identity. Although there have been reports studying the overall immunogenicity of S_{SL}, knowledge on the precise location of immunodominant determinants for S_{SL} is still lacking. In this study, using a series of truncated expressed S_{SL} fragments and S_{SL} specific mouse sera, we identified two immunogenic determinants for S_{SL}. Importantly, one of the two regions seems to be located in a region not shared by known immunogenic determinants of the S_{SARS}. This finding will be of potential use in future monitoring of SL-CoV infection in bats and spillover animals and in development of more effective vaccine to cover broad protection against this new group of coronaviruses.

Bat; SL-CoV; Immune; Vaccine

Since the first description of SARS-like coronavirus (SL-CoV) (Lau S K, et al., 2005; Li W, et al., 2005), there have been successive reports on the detection of this group of new coronaviruses in bats around the world (Lau S K, et al., 2010; Ren W, et al., 2006; Tang X C, et al., 2006; Yuan J, et al., 2010). A pathogenicity study using synthesized SL-CoV showed that it could cause disease in mice if it acquired a small fragment from SARS-CoV (Becker M M, et al., 2008). These works highlighted the potential for SL-CoV to spillover into non-bat mammals, including humans, and cause disease.

The SARS-CoV spike protein (S_{SARS}) is responsible for receptor binding and is also a major target of neutralizing antibodies (He Y, 2006). Based on the significant sequence difference between the SL-CoV spike protein (S_{SL}) and S_{SARS}, it is no surprising to find that sera from SL-CoV positive failed to neutralize SARS-CoV (Li W, et al., 2005). In our previous study, we found that SL-CoV infected bat sera could recognize a HIV-pseudovirus carrying the S_{SL} protein, but not a similar pseudovirus carrying a mutant S_{SL} protein with its receptor binding region replaced by that from the S_{SARS} protein (Zhou P, et al., 2009). From that study, we postulated that the major immunodominant neutralizing epitope may lie in this region of the S_{SL} protein.

Using five truncated expressed proteins and a panel of S_{SL}-specific mouse polyclonal and monoclonal antibodies, we identified two immunogenic determinants, one of which is located in a different region from those identified for S_{SARS}. This is the first report to identify immunogenic determinants on S_{SL}. The data presented here will be useful for future development of both diagnostics and vaccines against SL-CoV.

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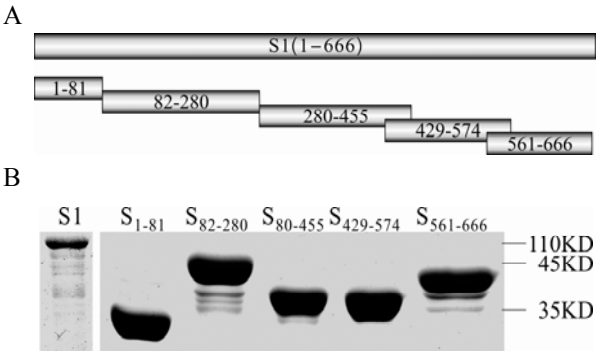


Fig. 1. Schematic diagram of S constructs and purified proteins generated in this report. A: The full-length SL-CoV S1 and the five truncated versions; the numbers shown below indicate the region (aa residue position) of each fragment. B: SDS-PAGE profile of the 6 purified proteins used in this study. The full-length S1 was expressed with MBP tag (42 kD) and the five truncated S1 were expressed in pET32a (with a 20 kD tag).

MATERIALS AND METHODS

Expression and purification of recombinant proteins

Six gene fragments covering the N-terminal S1 region of the S_{SL} gene, as shown in Fig. 1A, were obtained by PCR using a plasmid containing the full-length S gene (Li W, et al., 2005). Restriction enzyme sites were incorporated into each PCR primer pair, *Eco*R I in the forward primer and *Sal* I in the reverse primer, to facilitate cloning (primer sequences are available upon request). The resultant PCR fragments were purified, restriction digested and ligated into prokaryotic expression vector pET32a (Novagen) for the truncated proteins, and to pMAL-c2x vector (New England Biolabs, Inc.) for full-length S1. Plasmids were sequenced to make sure that the gene fragments are free of mutations and cloned in frame with vector encoded protein tag sequences.

The recombinant plasmids were transformed into *Escherichia coli* strain BL-21(DE3) (Novagen) and cultured at 37 °C until OD_{600} reached 0.6 to 0.8. Protein expression was induced for 6 h with 0.3 mmol/L IPTG (isopropyl-β-Dithiogalactopyranoside) at 30°C. Cells were harvested and resuspended in phosphate-buffered saline (PBS) and lysed by sonication after 3 cycles of freeze-thawing. The resulting lysates were centrifuged at 13,000×*rpm* for 15 min at 4 °C, and the clarified supernatants were collected and applied either to His-Bind column (Novagen) for the truncated S1 fragments or to Amylose Resin (New England Biolabs, Inc.) for full-length S1 protein according to the manufacturers' protocols. The purity of the recombinant proteins was analyzed by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE).

Production of antibodies

Mouse sera were prepared by DNA immunization using plasmids expressing the SL-CoV Rp3 S gene as described previously (Zhou P, et al., 2009). Monoclonal antibodies against SL-CoV S1 were made using published standard procedures (Evan G I, et al., 1985). Briefly, BALB/c mice were immunized with purified HIV/ Rp3-S pseudotyped virus produced in our previous work (Zhou P, et al., 2009). Spleen cells from immunized mice were harvested and fused with Sp2/0 myeloma cells. Cell culture supernatants from the hybridoma containing wells were screened by ELISA using HIV/ Rp3-S pseudovirus and purified Rp3 S1 protein as coating antigens. Positive clones were expanded and inoculated intraperitoneally into syngeneic mice using protocol approved by the Animal Ethics Committee at the Wuhan Institute of Virology. Ascites fluids were tapped and the titers of the monoclonal antibodies were determined by ELISA. To avoid cross reaction between the monoclonal antibodies and pET32a tag protein in Western blot, all monoclonal antibodies were pre-absorbed, before use, with control *E. coli* lysate containing pET32a encoded proteins.

Indirect ELISA

ELISA assays were performed under standard conditions. Briefly, 96-well microtiter plates were coated with purified recombinant proteins (50 to 100 ng/well) in 0.1 mol/L carbonate buffer (pH 9.3) over night at 4 °C. The plates were washed and blocked with 5% BSA in PBS-0.1% Tween 20, and then incubated with either mouse sera or monoclonal antibodies for 1 h at 37 °C. Bound antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse IgG (LingFei Tech., Wuhan, China) with a dilution at 1:4000. Color development was conducted using 3,3,5,5, tetramethylbenzidine (TMB) and the absorbance at 450 nm was determined after the reaction was stopped with 2 mol/L H_2SO_4. All washes were carried out five times using PBS-0.1% Tween (2 min/wash), and all antibodies were diluted using 0.5% BSA in PBS-0.1% Tween. An appropriate negative control was included in every step.

SDS-PAGE and Western blot

Proteins were separated on 12% SDS-PAGE gels followed by Coomassie blue staining using standard procedures (Wilson C M, 1983). For Western blot analysis, separated proteins were transferred to polyvinyldifluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were then blocked with 5% skim milk in

PBS-0.1% Tween for 1h at 37°C. After three washes, the membranes were incubated overnight at 4°C with mouse sera at 1:1000 or monoclonal antibodies at 1:250. Bounded antibodies were detected for 1h at 37°C with alkaline phosphatase-conjugated Affinipure goat anti-mouse IgG (Proteintech Group, Inc., Wuhan, China) at 1:2000, and developed using the TMB membrane peroxidase substrate system. The developed membranes were washed and air dried.

Sequence alignment and structure modeling

The amino acid sequences of S proteins were obtained from NCBI (GeneBank accession no. YP_001382361.1 for Rp3 S and AAP41037.1 for Tor2 S). Sequence alignment was done using Clustal W (Thompson *et al.,* 1994). Protein structures were modeled using CPHmodels 3.0 (Lund et al 2002) and PyMOL 1.2.8 (DeLano scientific).

RESULTS

Expression and purification of recombinant proteins

Six recombinant proteins, including full-length S1 and five truncated fragments, were successfully expressed in *E. coli*. All of them were soluble, which greatly facilitated affinity purification based on their fused protein tags. After purification, the purity was analyzed by SDS-PAGE followed by Coomassie blue staining. A purity of 90% or more was achieved for all of the recombinant proteins (Fig. 1B).

Identification of immunogenic determinants using truncated recombinant S_{SL} fragments

Five purified S1 fragments were tested by ELISA against sera from four mice immunized with plasmid DNA encoding full-length Rp3-S. Fig. 2A summarizes the resulting immune responses in the four immunized mice. Most of the sera reacted against $S_{280-455}$ (4/4) and $S_{561-666}$ (3/4), while one animal reacted against $S_{429-574}$ (1/4), and none reacted against S_{1-82} (0/4) or S_{82-280} (0/4).

Mapping of immunogenic regions recognized by Rp3 S monoclonal antibodies (mAb)

HIV/Rp3-S pseudotyped virus (Ren W, et al., 2008) was used to immunize mouse for production of monoclonal antibodies against S_{SL}. Successful production of antibodies was confirmed by ELISA against both HIV/Rp3-S and recombinant full-length S1 proteins, demonstrating that the mAbs reacted with the Rp3 S protein but not to the HIV pseudovirus backbone proteins (data not shown). As

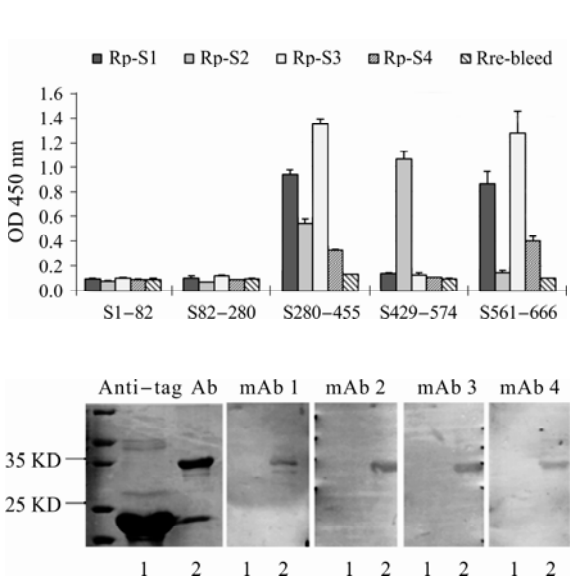


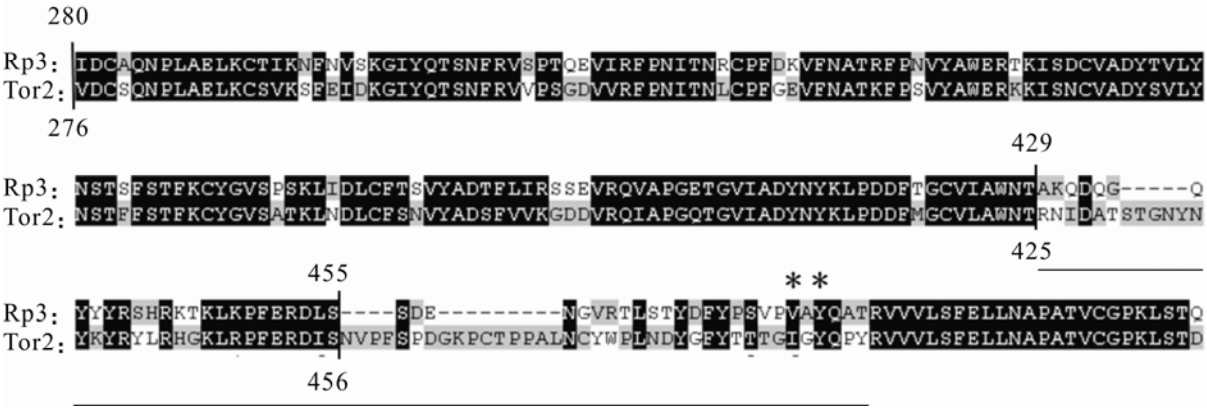
Fig. 2. Mapping of the immunogenic determinants of the S1 protein. A: ELISA analysis of reactivity between the five truncated proteins and DNA immunized Rp3 S sera. Combined pre-bleed sera were used as a negative control. Data are presented as means± SE (standard error). The bars indicate three separate experiments. B: Western blot analysis of reactivity between the $S_{280-455}$ and four monoclonal antibodies. Lane 1: control protein expressed from the vector alone; Lane 2: recombinant $S_{280-455}$ protein.

shown by Western blot analysis, all four mAbs reacted with the $S_{280-455}$ protein (Fig. 2B), but not the pET32a tag protein or the other four truncated fragments (data not shown), providing further evidence that aa 280-455 is an immunogenic determinants. However, due to lacking the susceptible cell lines for SARS-like CoV Rp3, we were unable to test neutralization ability of these four mAbs.

DISCUSSION

Although there have been many studies to characterize the immunodominant determinants in SARS-CoV spike protein, S_{SARS} (He Y, 2006; Hua R, et al., 2004), which serves as an important target for vaccine design, there has been no report to date characterizing the immunogenic determinants for the bat SL-CoV spike protein, S_{SL}. SL-CoV, which was first identified in 2005 (Lau S K, et al., 2005; Li W, et al., 2005), may have the potential to spillover to humans through a minimal change in its spike protein (Lau S K, et al., 2010). This prediction was confirmed in a recent study where a recombinant SL-CoV containing a very small fragment of the SARS-CoV S gene was able to infect and cause disease in mice, further highlighting its potential for pathogenicity in humans (Becker

A



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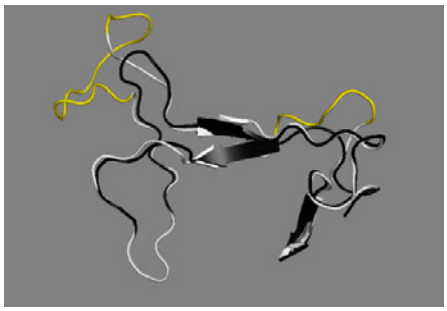

Fig. 3. Comparison of the aa 280-504 region of S_{SL} with the corresponding region of S_{SARS} in SARS-CoV Tor2 S (aa 276-518). A: Alignment of the aa sequences of the two regions. The underlined region indicates the receptor binding motif (RBM) in Tor2 S. The aa residue numbers for Rp3 S and Tor2 S are provided above or beneath the sequence alignment, respectively. The key residues at aa 489 and 491 in S_{SARS} are highlighted by asterisks. B: Structural modeling of the receptor binding motif (RBM) of Tor2 S (white) and the corresponding region in Rp3 S (black). The region present in Tor2 S but absent in Rp3 S was highlighted in yellow.

M M, et al., 2008). In view of this, defining the immunogenic determinants of S_{SL} is necessary for both the detection of SL-CoV in bats and future vaccine design.

In the absence of a live SL-CoV, we have embarked a study to determine immunodominant determinants of S_{SL} using mouse sera and monoclonal antibodies raised against a pseudovirus expressing the full length S_{SL} protein. From a series of recombinant truncated S_{SL} expressed in this study, we were able to show, by both ELISA and Western blot, that $S_{280-455}$ and $S_{561-666}$ represent two immunogenic determinants in mice. The region covering aa 280-455 was the more immunogenic of the two since it was recognized not only by the polyclonal mouse sera, but also by all four monoclonal antibodies. It is worth to mention that the S1-81 and S82-280 may also contain conformational immunogenic determinants yet not be identified by the bacteria proteins in this study. It is possible that the use of recombinant full-length S1 protein as selection antigen will result into the loss of valuable monoclonals. Further screening of more immunogenic determinants needs to be performed with protein expressed in eukaryotic cells.

From a previous report we know that aa 528-635 of S_{SARS} is a major immunodominant determinants (He Y, et al., 2004), and S_{SL} shares very high sequence identity in this region (Li W, et al., 2005). In view of this, it is not surprising that $S_{561-666}$ of S_{SL} also demonstrated the immunogenic in mouse. However, the receptor binding domain (RBD) of S_{SL} has low sequence identity to that of S_{SARS}, especially in the critical receptor binding motif (RBM) region (Fig. 3A). The RBD region of S_{SARS} was known to be a major target of neutralizing antibodies (He Y, 2006; He Y, et al., 2004). The corresponding region of S_{SL} also showed high level of immunogenicity, as reported previously (Zhou P, et al., 2009). However, a subtle difference was observed from this study. Although residues in the RBM region were reported to be a hotspot for neutralizing antibody production in S_{SARS} (Zhu Z, et al., 2007), the corresponding region (underlined in Fig. 3A) in S_{SL} failed to react with any of the four monoclonal antibodies and only reacted with one of the four mouse sera. From a 3D modeling of the RBM region (Fig. 3B) for the two S proteins, it is evident that the two structures are almost identical except for a few very minor differences. Interestingly, several predicted hotspots for antibody targeting in different SARS-CoV strains, especially those covering the residues 489 and 491 (Zhu Z, et al., 2007), are highly conserved in S_{SL} both in sequence and predicted structure. But these regions seemed to be non-immunogenic in mice from this study. This could imply a subtle, but important difference in the location of immunodominant determinants between the two S proteins. However, it has to be said that the current study

was based on an immunization study in mice, rather than an infection study. Further investigation is required before we can fully assess the potential immunogenicity differences of the different bat SL-CoV S proteins.

Nevertheless, it is worth noting that the antibodies generated in this study will be a useful tool in many aspects. For example, combining the $S_{280\text{-}455}$ specific mAbs and the more cross reactive N protein-specific antibodies, we can design tests that could simultaneously detect SARS-related coronaviruses as well as determine potential recombinant events in the S genes. This will be especially beneficial for the long term monitoring of SL-CoVs in bats, which is being actively pursued by many groups around the world (Lau S K, et al., 2010).

In summary, this work represents a first attempt to determine the immunogenic determinants for S_{SL} in comparison to S_{SARS}. The knowledge and reagents generated from the current study will facilitate ongoing SL-CoV surveillance and research, including the development of better diagnostics and broad spectrum vaccines against this new group of coronaviruses.

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References

Becker M M, Graham R L, Donaldson E F, Rockx B, Sims A C, Sheahan T, Pickles R J, Corti D, Johnston R E, Baric R S, and Denison M R. 2008. **Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice**. Proc Natl Acad Sci U S A, 105: 19944-19949.

Evan G I, Lewis G K, Ramsay G, and Bishop J M. 1985. **Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product**. Mol Cell Biol, 5: 3610-3616.

He Y. 2006. **Immunogenicity of SARS-CoV: the receptor-binding domain of S protein is a major target of neutralizing antibodies**. Adv Exp Med Biol, 581: 539-542.

He Y, Zhou Y, Wu H, Luo B, Chen J, Li W, and Jiang S. 2004. **Identification of immunodominant sites on the spike protein of severe acute respiratory syndrome (SARS) coronavirus: implication for developing SARS diagnostics and vaccines**. J Immunol, 173: 4050-4057.

He Y, Zhou Y, Liu S, Kou Z, Li W, Farzan M, and Jiang S. 2004. **Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine**. Biochem Biophys Res Commun, 324: 773-781.

Hua R, Zhou Y, Wang Y, Hua Y, and Tong G. 2004. **Identification of two antigenic epitopes on SARS-CoV spike protein**. Biochem Biophys Res Commun, 319: 929-935.

Lau S K, Woo P C, Li K S, Huang Y, Tsoi H W, Wong B H, Wong S S, Leung S Y, Chan K H, and Yuen K Y. 2005. **Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats**. Proc Natl Acad Sci U S A, 102: 14040-14045.

Lau S K, Li K S, Huang Y, Shek C T, Tse H, Wang M, Choi G K, Xu H, Lam C S, Guo R, Chan K H, Zheng B J, Woo P C, and Yuen K Y. 2010. **Ecoepidemiology and complete genome comparison of different strains of severe acute respiratory syndrome-related Rhinolophus bat coronavirus in China reveal bats as a reservoir for acute, self-limiting infection that allows recombination events**. J Virol, 84: 2808-2819.

Li W, Shi Z, Yu M, Ren W, Smith C, Epstein J H, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, Daszak P, Eaton B T, Zhang S, and Wang L F. 2005. **Bats are natural reservoirs of SARS-like coronaviruses**. Science, 310: 676-679.

Ren W, Qu X, Li W, Han Z, Yu M, Zhou P, Zhang S Y, Wang L F, Deng H, and Shi Z. 2008. **Difference in receptor usage between severe acute respiratory syndrome (SARS) coronavirus and SARS-like coronavirus of bat origin**. J Virol, 82: 1899-1907.

Ren W, Li W, Yu M, Hao P, Zhang Y, Zhou P, Zhang S, Zhao G, Zhong Y, Wang S, Wang L F, and Shi Z. 2006. **Full-length genome sequences of two SARS-like coronaviruses in horseshoe bats and genetic variation analysis**. J Gen Virol, 87: 3355-3359.

Tang X C, Zhang J X, Zhang S Y, Wang P, Fan X H, Li L F, Li G, Dong B Q, Liu W, Cheung C L, Xu K M, Song W J, Vijaykrishna D, Poon L L, Peiris J S, Smith G J, Chen H, and Guan Y. 2006. **Prevalence and genetic diversity of coronaviruses in bats from China**. J Virol, 80: 7481-7490.

Wilson C M. 1983. **Staining of proteins on gels: comparisons of dyes and procedures**. Methods Enzymol, 91: 236-247.

Yuan J, Hon C C, Li Y, Wang D, Xu G, Zhang H, Zhou P, Poon L L, Lam T T, Leung F C, and Shi Z. 2010. **Intraspecies diversity of SARS-like coronaviruses in Rhinolophus sinicus and its implications for the origin of SARS coronaviruses in humans**. J Gen Virol, 91: 1058-1062.

Zhou P, Han Z, Wang L F, and Shi Z. 2009. **Immunogenicity difference between the SARS coronavirus and the bat SARS-like coronavirus spike (S) proteins**. Biochem Biophys Res Commun, 387: 326-329.

Zhu Z, Chakraborti S, He Y, Roberts A, Sheahan T, Xiao X, Hensley L E, Prabakaran P, Rockx B, Sidorov I A, Corti D, Vogel L, Feng Y, Kim J O, Wang L F, Baric R, Lanzavecchia A, Curtis K M, Nabel G J, Subbarao K, Jiang S, and Dimitrov D S. 2007. **Potent cross-reactive neutralization of SARS coronavirus isolates by human monoclonal antibodies**. Proc Natl Acad Sci U S A, 104: 12123-12128.