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Antigenicity and receptor-binding ability of recombinant SARS coronavirus spike protein

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Abstract

Severe acute respiratory syndrome (SARS) is an emerging infectious disease associated with a novel coronavirus and causing worldwide outbreaks. SARS coronavirus (SARS-CoV) is an enveloped RNA virus, which contains several structural proteins. Among these proteins, spike (S) protein is responsible for binding to specific cellular receptors and is a major antigenic determinant, which induces neutralizing antibody. In order to analyze the antigenicity and receptor-binding ability of SARS-CoV S protein, we expressed the S protein in Escherichia coli using a pET expression vector. After the isopropyl-β-D-thiogalactoside induction, S protein was expressed in the soluble form and purified by nickel-affinity chromatography to homogeneity. The amount of S protein recovered was 0.2–0.3 mg/100 ml bacterial culture. The S protein was recognized by sera from SARS patients by ELISA and Western blot, which indicated that recombinant S protein retained its antigenicity. By biotinylated ELISA and Western blot using biotin-labeled S protein as the probe, we identified 130-kDa and 140-kDa proteins in Vero cells that might be the cellular receptors responsible for SARS-CoV infection. Taken together, these results suggested that recombinant S protein exhibited the antigenicity and receptor-binding ability, and it could be a good candidate for further developing SARS vaccine and anti-SARS therapy.

Keywords: SARS; Coronavirus; Spike; Expression; Antigenicity; Receptor-binding

Severe acute respiratory syndrome (SARS) is a newly emerging human disease, resulting globally in 774 deaths from 8098 probable cases [1]. A novel coronavirus has been identified as the etiological agent of SARS and designated as SARS coronavirus (SARS-CoV) after tests of causation according to Koch’s postulates, including monkey inoculation [2–4]. SARS-CoV can infect African green monkey kidney (Vero E6) cells and cause a similar disease in cynomolgus macaques (Macaca fascicularis) [2,3]. The full-length genome sequence of SARS-CoV has been elucidated within weeks after the identification of this novel pathogen [5,6]. SARS-CoV contains a single-stranded plus-sense RNA genome about 30 kb in length that has a 5’-cap structure and a 3’-polyadenylation tract. The genomic organization is typical of coronaviruses, having five major open reading frames (ORFs) that encode the replicate polyproteins; the spike (S), envelope (E), and membrane (M) glycoproteins; and the nucleocapsid protein (N) in the same order as those of other coronaviruses [5–7].

When coronaviruses enter cells, the 5’-region of viral genome is translated into a large polypeptide that is cleaved by viral-encoded proteases to release RNA-dependent RNA polymerase and adenosine triphosphatase/helicase. These proteins, in turn, are responsible for replicating the viral genome as well as generating nested transcripts that are used in the synthesis of viral proteins. Viral membrane proteins, including S and M are inserted into the endoplasmic reticulum (ER), while RNA genome assembles with the N protein. This RNA-protein complex then associates with M proteins and buds into the lumen of the ER. The virus particles then migrate through the Golgi complex and exist in the cells
by exocytosis [7]. The first step in viral infection is the binding of viral proteins to certain cellular receptors. So far, the S protein of coronavirus is considered as the site of viral attachment to the host cells [8,9].

The S proteins of coronaviruses are large type I membrane glycoprotein projections from viral envelope [10]. S proteins are responsible for both binding to receptors on host cells and for membrane fusion [11,12]. S proteins also contain important virus-neutralizing epitopes that elicit neutralizing antibody in the host species [13,14]. Furthermore, mutations in this gene dramatically affect the virulence, pathogenesis, and host cell tropism [15–17]. These results suggested that S protein is a good candidate for vaccine because neutralizing antibodies are directed against S. Moreover, S protein is also a good target for antiviral therapies because blockade of binding of S protein to cellular receptor can prevent virus entry. Therefore, in this study, we expressed the SARS-CoV S protein in Escherichia coli (E. coli). The antigenicity and receptor-binding ability of recombinant S protein were further analyzed.

**Materials and methods**

*Construction of recombinant plasmids.* SARS-CoV RNA was provided by Department of Medical Technology, China Medical University Hospital. SARS-CoV S gene was kindly provided by Dr. P.J. Chen. Briefly, the RNA was reverse transcribed into cDNA using SARS-M2 primer (5′-GGAATTCGCCAAACATACCAAGGCC-3′) and Superscript III (Invitrogen) according to the manufacturer’s protocol. The 3580-bp DNA fragment was then amplified from the cDNA template by polymerase chain reaction (PCR) using PfuUter DNA polymerase (Stratagene) with SARS-P1 primer (5′-CGGGATCTAGGGTAGGTAGT GACCTTGACC-3′) and SARS-M2 primer. The PCR profile was as follows: one cycle at 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min. The amplified cDNA fragments were cleaved by EcoRI and BamHI, and the 3580-bp fragments were then inserted into pET-28b(+) (Novagen) to create the pET-spike expression plasmid.

**DNA sequencing.** DNA sequencing was performed on double-stranded plasmids by dyeode chain termination with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and analyzed by ABP Prism 310 (Applied Biosystems). Dimethyl sulfoxide was introduced to the reaction to a final concentration of 5% to diminish the secondary structure. The primer used in the sequencing reaction was T7, SARS-P2 (5′-CGGGATCTCTTATTACACTGGAACAAATGC-3′), T7 terminator, SARS-M2, SARS-M3 (5′-TTGATATAGAACAGC AACCTTACATGAAGC-3′), or SARS-M1 (5′-GGAATTCCTGACCCACCCCAAAAGAGC-3′). DNA was sequenced on both strands of at least two repeats of cloned DNA fragments.

Expression and purification of recombinant SARS-CoV S protein. Recombinant protein was expressed in E. coli BL21(DE3)pLysS strain by transforming the pET-spike to produce an N-terminal fusion with six histidine residues. The expression and purification of recombinant S protein were performed as described previously with modification [18]. Briefly, cells were grown in 100 ml Luria–Bertani broth agitated at 37°C until OD 600 reached 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was then added to a final concentration of 0.5 mM and the cells were pelleted 2 h after induction. The cell pellet was washed twice with ice-cold binding buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, and 5 mM imidazole) and resuspended in 5 ml ice-cold binding buffer containing 8 M urea. The resuspended pellet was incubated at 4°C with shaking for 2 h. After sonication, the suspension was centrifuged at 16,000g for 30 min at 4°C. The resulting supernatant was applied on nickel-affinity chromatography with 8 M urea present throughout the procedure. The protein in the final column eluate was dialyzed overnight against phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na 2HPO 4, and 1.4 mM KH 2PO 4) and stored at −70°C until further analysis. Protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and was quantified with a Bradford assay (Bio-Rad).

Cells and cell extracts. Human lung carcinoma cell (A-549), human liver cell (Chang liver), African green monkey kidney cells (Vero E6, Vero), and human monocyte (U-397) were purchased from Bioresources Collection and Research Center (Hsinchu, Taiwan). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C and 5% CO 2 in air. The cell extracts were prepared by washing the cells once with ice-cold PBS and lysing the cells by lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris–HCl, pH 8.0, and 20 μM phenylmethylsulfonyl fluoride). After a 30-min-incubation on ice, the cell lysate was collected by centrifugation. Protein was quantified with a Bradford assay and stored at −30°C until further analysis.

![Fig. 1. Genome organization of SARS-CoV and putative region of SARS-CoV S protein. (A) Overall organization of the 29,727-nt SARS-CoV. Predicted ORF 1a and ORF 1b encode replicate 1A and 1B, respectively. S, E, M, and N genes encode spike, envelope, membrane, and nucleocapsid proteins, respectively. S gene is situated at 21,492–25,259 nucleotide in the SARS-CoV genome. (B) Predicted functional regions of SARS-CoV S protein. The upper diagram represents the 1255-residue S protein. Regions spanning 1–13 and 1196–1255 are putative signal peptide, and transmembrane and cytoplasmic regions, respectively. Putative receptor-binding site is located at residue 757–761.](image-url)
Fig. 2. Multiple alignment of coronavirus S proteins. Amino acid sequences of S proteins from human respiratory coronavirus (HCoV-229E) (GenBank Accession No. P15423), porcine transmissible gastroenteritis virus (TGEV) (GenBank Accession No. CAA37285), feline infectious peritonitis virus (FIPV) (GenBank Accession No. P10033), human respiratory coronavirus (HCoV-OC43) (GenBank Accession No. AAA03055), murine hepatitis virus (MHV) (GenBank Accession No. AAC56567), porcine hemagglutinating coronavirus (HEV) (GenBank Accession No. AAM77000), and bovine coronavirus (BCoV) (GenBank Accession No. AAF25499) were aligned with those of SARS-CoV by PileUp program. Residues that are similar in all coronaviruses are shown in uppercase. The locations of signal peptide, transmembrane and cytoplasmic regions of SARS-CoV S protein are underlined. The putative receptor-binding site of SARS-CoV S protein and antigenic sites of TGEV are shown in bold.
Biotinylation of recombinant S protein. Recombinant S protein was mixed with Sulfo-NHS-biotin (Pierce) in a ratio of ten to one. After a 2-h-incubation on ice, the unincorporated biotin was removed by centricon-10 (Amicon) and the biotinylated S protein was stored at 4°C until further analysis. Sulfo-NHS-biotin should be prepared freshly by dissolving in DDW.

Western blot analysis. Proteins (5 μg) were separated by 10% SDS–PAGE and the protein bands were then transferred electrophoretically to nitrocellulose membrane (Amersham Pharmacia Biotech). Membrane was blocked in blocking buffer (20 mM Tris–HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20, and 5% skim milk) and probed with rabbit anti-spike (residue 511–993) polyclonal antibody or human anti-SARS sera at room temperature for 1 h. The bound antibody was detected with horseradish peroxidase-conjugated secondary antibody (Sigma) followed by chemiluminescence (ECL system, Amersham) and exposed by X-ray films. Rabbit anti-spike (residue 511–993) polyclonal antibody was prepared by immunizing rabbit with truncated spike and was provided kindly by Dr. P.J. Chen. Human anti-SARS sera were collected from SARS patients and provided by Dr. L.K. Chen.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc) were coated at 4°C overnight with 50 μl of 10 ng/μl proteins, which was diluted in 0.05 M carbonate buffer (pH 9.6). The wells were rinsed with 200 μl washing buffer (0.5% Tween 20 in PBS) and blocked with 200 μl blocking buffer (5% bovine serum albumin (BSA) in washing buffer) by incubating at 37°C for 30 min. The absorbed protein in each well was challenged with 50 μl diluted human anti-SARS sera and incubated at 37°C for 1 h. Following three washes, chromogenic substrate was added to each well and incubated at 37°C for 15 min. The absorbance was read at 405 nm in an ELISA plate reader.

Results and discussion

The SARS-CoV genome is approximately 29.7 kb long and contains five major ORFs flanked by 5′ and 3′ untranslated regions of 265 and 342 nucleotides, respectively (Fig. 1A) [5,6]. The predicted S gene is located from nucleotide 21,492 to 25,259 in the SARS-CoV genome. In order to clone S gene, the 3580-bp DNA fragment containing whole S gene was amplified by reverse transcription-PCR and inserted into prokaryotic vector pET-28(+). The deduced amino acid sequence of S protein is shown in Fig. 2. (continued)
domain and a cytoplasmic tail rich in cysteine residues. Twenty-three potential N-linked glycosylation sites were predicted among SARS-CoV S protein. Together these data predicted that SARS-CoV S protein is a type I membrane glycoprotein with the N-terminus and the majority of the protein (residue 14–1195) on the outside of virus particle, in agreement with other coronavirus S protein data (Fig. 1B).

Multiple alignment and phylogenetic analysis among S proteins of mammalian coronaviruses, which cause respiratory infection, were further performed by PileUp program of Genetics Computer Group (GCG) (Figs. 2 and 3). Alignment of these sequences produced a low level of similarity (20–27% pairwise amino acid identity) between the predicted amino acid sequence of SARS-CoV S protein and other coronavirus S proteins. Phylogenetic analysis showed that the species formed monophyletic clusters consistent with established taxonomic groups. However, SARS-CoV S protein sequences segregated into a well-resolved branch, indicating that SARS-CoV S protein is not closely related to any of the previously characterized coronavirus S proteins. Although overall sequence conservation was low, the C-terminus, consisting of a transmembrane domain and a cytoplasmic tail, was highly conserved. Putative cellular receptor-binding sites (residue 757–761) of SARS-CoV S protein [19] were not conserved compared with other coronaviruses. The antigenic sites of porcine transmissible gastroenteritis virus S protein [20,21] were also varied among these coronaviruses. These data suggested that comparison of primary amino acid sequences does not provide insight into the receptor-binding specificity or antigenic properties of SARS-CoV S protein.

By analyzing the primary structure of SARS-CoV S protein, we tried to predict the putative antigenic regions of S protein. The hydrophilicity, surface probability, and chain flexibility of SARS-CoV S protein were calculated by PeptideStructure program of GCG according to Kyte and Doolittle plots, Emini prediction, and Karplus and Schulz prediction, respectively (Fig. 4A) [22–24]. The antigenicity of SARS-CoV S protein was further analyzed based on three aforementioned criteria, and the regions in which antigenic index exceeds 1.3 are shown in Fig. 4B. Most of the putative antigenic sites were located at outer membrane regions, in which residue 1–100 and 401–500 exhibited the highly antigenic potential. Because most T cell and B cell epitopes contain a sequence of 6–20-amino acids [25,26], we further analyzed the antigenicity using a window of 15 residues. Residue 12–50, 426–456, 478–494, 541–564, and 922–1118 of SARS-CoV S protein displayed the highly antigenic potential. These data suggested that these regions might be good candidates for developing SARS peptide vaccine.

In order to study the antigenicity and receptor-binding ability of SARS-CoV S protein, we expressed and purified the full-length recombinant SARS-CoV S protein from E. coli. We expressed the S protein from E. coli BL21(DE3)pLysS strain transformed with a pET plasmid carrying S gene. After induction with IPTG, a product with 138-kDa was observed by SDS–PAGE.
The amount of induced S protein was consistent when the bacteria were refreshed to 0.45–0.6 OD_{600} and the recombinant S protein was expressed in the soluble form in bacterial cells. The soluble S protein was further purified by affinity chromatography using His-Bond resin. No detectable S protein was purified, suggesting that the histidine tag is folded into the interior of S protein (data not shown). We therefore denatured the S protein by urea, purified by nickel-affinity chromatography, and renatured the protein by dialysis. The amount of recombinant S protein recovered was approximately 0.2–0.3 mg/100 ml of bacterial culture (Fig. 6).

To analyze the antigenicity of recombinant S protein, we performed Western blot and ELISA using sera from SARS patients or from spike-immunized rabbits. Fig. 7
shows that recombinant S was detectable by both sera in both assays. Additionally, truncated S protein (residue 511–993) exhibited the similar antigenicity compared with full-length spike (residue 1–1255). These results indicated that recombinant S protein remains its antigenicity that could be recognized by sera from SARS patients.

The receptor-binding ability of recombinant S protein was analyzed by biotinylated ELISA and Western blot. Several reports indicated that SARS-CoV could have succeeded in growing progeny virus in Vero cells [3,5,6]. The BSA and Vero cell extracts were therefore
coated on ELISA plates and challenged with biotin-labeled S protein. The binding ability of S protein to cell extract was evaluated by OD_{405} value in ELISA. Fig. 8A shows that the binding ability of S protein to BSA was very low. However, S protein significantly bound Vero cell extracts with the OD_{405} exceeding 0.9. These results proved the specificity of biotinylated ELISA in analyzing the receptor-binding ability of S protein. It also suggested that recombinant S protein could be served as a probe to analyze the cellular receptors involved in virus attachment.

To further identify the potential cellular receptors for SARS-CoV attachment, we performed biotinylated Western blot. The cell extracts were separated by SDS-PAGE, transferred to membrane, and detected by biotin-labeled S protein. Fig. 8B shows that recombinant S protein interacted with several cellular proteins in different cell types. By comparison of protein patterns of different cell types, we found that two proteins with molecular masses of 130 and 140 kDa were detectable in Vero cells instead of other cell types. It is now known that Vero cell is the only cell line that could be infected by SARS-CoV [3]. These results suggested that 130-kDa and 140-kDa polypeptides in Vero cells might be the cellular receptors responsible for SARS-CoV S protein binding. The elucidation of amino acid sequences of these proteins is now proceeded.

In this study, we cloned, expressed, and purified the SARS-CoV S protein from E. coli. The recombinant S protein was expressed in soluble form in bacterial cells, and the amount of protein recovered was 0.2–0.3 mg/100 ml bacterial culture. The S protein was recognized by sera from SARS patients, indicating that recombinant S protein retained its antigenicity. By biotinylated ELISA and Western blot using recombinant S protein as the probe, we identified that 130-kDa and 140-kDa proteins might be the cellular receptors responsible for SARS-CoV infection. These results suggested that S protein could be a good candidate for further developing SARS vaccine and anti-SARS therapy.

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References

[1] WHO. Cumulative number of reported probable cases of severe acute respiratory syndrome (SARS), www.who.int/csr/sars/country/table2003_09_23/en/ (accessed September 23, 2003).

[2] R.A. Fouchier, T. Kuiken, M. Schutten, G. van Amerongen, G.J.J. van Doornum, B.G. van den Hoogen, M. Peiris, W. Lim, K. Stohr, A.D.M.E. Osterhaus, Koch’s postulates fulfills for SARS virus, Nature 423 (2003) 240.

[3] T.G. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A. Ling, C.D. Humphrey, W. Shieh, J. Guaner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, the SARS working group, A novel coronavirus associated with severe acute respiratory syndrome, N. Engl. J. Med. 348 (2003) 1953–1966.

[4] J.S.M. Peiris, S.T. Lai, L.M.M. Poon, Y. Guan, L.Y.C. Yam, W. Lim, J. Nicholls, W.K.S. Yee, W.W.Y. Chan, M.T. Cheung, V.C.C. Cheng, K.H. Chan, D.N.S. Tsang, R.W.H. Yung, T.K. Ng, K.Y. Yuen, members of the SARS study group, Coronavirus as a possible cause of severe acute respiratory syndrome, Lancet 361 (2003) 1319–1325.

[5] L.M. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A. Ling, C.D. Humphrey, W. Shieh, J. Guaner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, the SARS working group, A novel coronavirus associated with severe acute respiratory syndrome, Nature 423 (2003) 240.

[6] J.S.M. Peiris, S.T. Lai, L.M.M. Poon, Y. Guan, L.Y.C. Yam, W. Lim, J. Nicholls, W.K.S. Yee, W.W.Y. Chan, M.T. Cheung, V.C.C. Cheng, K.H. Chan, D.N.S. Tsang, R.W.H. Yung, T.K. Ng, K.Y. Yuen, members of the SARS study group, Coronavirus as a possible cause of severe acute respiratory syndrome, Lancet 361 (2003) 1319–1325.

[7] L.M. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A. Ling, C.D. Humphrey, W. Shieh, J. Guaner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, the SARS working group, A novel coronavirus associated with severe acute respiratory syndrome, Nature 423 (2003) 240.

[8] J.S.M. Peiris, S.T. Lai, L.M.M. Poon, Y. Guan, L.Y.C. Yam, W. Lim, J. Nicholls, W.K.S. Yee, W.W.Y. Chan, M.T. Cheung, V.C.C. Cheng, K.H. Chan, D.N.S. Tsang, R.W.H. Yung, T.K. Ng, K.Y. Yuen, members of the SARS study group, Coronavirus as a possible cause of severe acute respiratory syndrome, Lancet 361 (2003) 1319–1325.

[9] L.M. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A. Ling, C.D. Humphrey, W. Shieh, J. Guaner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, the SARS working group, A novel coronavirus associated with severe acute respiratory syndrome, Nature 423 (2003) 240.

[10] L.M. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A. Ling, C.D. Humphrey, W. Shieh, J. Guaner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, the SARS working group, A novel coronavirus associated with severe acute respiratory syndrome, Nature 423 (2003) 240.

[11] L.M. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A. Ling, C.D. Humphrey, W. Shieh, J. Guaner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, the SARS working group, A novel coronavirus associated with severe acute respiratory syndrome, Nature 423 (2003) 240.

[12] L.M. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A. Ling, C.D. Humphrey, W. Shieh, J. Guaner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, the SARS working group, A novel coronavirus associated with severe acute respiratory syndrome, Nature 423 (2003) 240.
coronaviruses: analysis using monoclonal antibodies to JHM (MHV-4) virus, Virology 131 (1983) 296–307.

[15] J.K. Fazakerley, S.E. Parker, F. Bloom, M.J. Buchmeier, The V5A13.1 envelope glycoprotein deletion mutant of mouse hepatitis virus type-4 is neuroattenuated by its reduced rate of spread in the central nervous system, Virology 187 (1992) 178–188.

[16] I. Leparc-Goffart, S.T. Hingley, M.M. Chua, J. Phillips, E. Lavi, S.R. Weiss, Targeted recombination within the spike gene of murine coronavirus mouse hepatitis virus-A59: Q159 is a determinant of hepatotropism, J. Virol. 72 (1998) 9628–9636.

[17] C.M. Sanchez, A. Izeta, J.M. Sanchez-Morgado, S. Alonso, I. Sola, M. Balasch, J. Plana-Duran, L. Enjuanes, Targeted recombination demonstrates that the spike gene of transmissible gastroenteritis coronavirus is a determinant of its enteric tropism and virulence, J. Virol. 73 (1999) 7607–7618.

[18] C.Y. Hsiang, T.Y. Ho, C.H. Hsiang, T.J. Chang, Recombinant pseudorabies virus DNase exhibits a RecBCD-like catalytic function, Biochem. J. 330 (1998) 55–59.

[19] X. Yu, C. Luo, J. Lin, P. Hao, Y. He, Z. Guo, L. Qin, J. Su, B. Liu, Y. Huang, P. Nan, C. Li, B. Xiong, X. Luo, G. Zhao, G. Pei, K. Chen, X. Shen, J. Shen, J. Zou, W. He, T. Shi, Y. Zhong, H. Jiang, Y. Li, Putative hAPN receptor binding sites in SARS-CoV spike protein, Acta Pharmacol. Sin. 24 (2003) 481–488.

[20] M. Mechin, M. der Vartanian, C. Martin, The major subunit C1pG of Escherichia coli CS31A fibrillae as an expression vector for different combinations of two TGEV coronavirus epitopes, Gene 179 (1996) 211–218.

[21] C. Smerdou, I.M. Anton, J. Plana, R. Curtiss, L. Enjuanes, A continuous epitope from transmissible gastroenteritis virus S protein fused to E. coli heat-labile toxin B subunit expressed by attenuated Salmonella induces serum and secretory immunity, Virus Res. 41 (1996) 1–9.

[22] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.

[23] E.A. Emini, J.V. Hughes, D.S. Perlow, J. Boger, Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide, J. Virol. 55 (1985) 836–839.

[24] P.A. Karplus, G.E. Schulz, Prediction of chain flexibility in proteins. A tool for the selection of peptide antigens, Naturwissenschaften 72 (1985) 212–213.

[25] A. Townsend, J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, A.J. McMichael, The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides, Cell 44 (1986) 959–968.

[26] H.M. Geysen, S.J. Rodda, T.J. Mason, G. Tribbick, P.G. Schoofs, Strategies for epitope analysis using peptide synthesis, J. Immunol. Methods 102 (1987) 259–274.