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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 replicase 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

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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 PfuUlter DNA polymerase (Stratagene) with SARS-P1 primer (5′-CGGGATCCTAGTGGTAGT 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 S cDNA fragments were cleaved by *Eco*RI and *Bam*HI, 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 dideoxy 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′-CGGGATCCTAATTACACCTGGAACAAATGC-3′), T7 terminator, SARS-M2, SARS-M3 (5′-TTGATATAGAACAGC AACTTCAGATGAAGC-3′), or SARS-M1 (5′-GGAATTCCACTTA CACCCCCAAAAGAGC-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 16,000*g* 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.

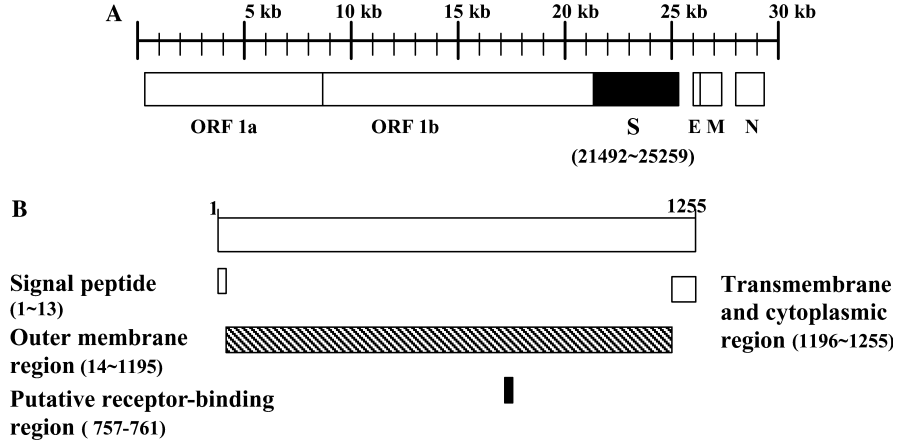

Fig. 1. Genome organization of SARS-CoV and putative region of SARS-CoV S protein. (A) Overall organization of the 29,727-nt SARS-CoV. Predicted ORF 1a and ORF 1b encode replicase 1A and 1B, respectively. S, E, M, and N genes encode spike, envelope, membrane, and nucleocapsid proteins, respectively. 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.

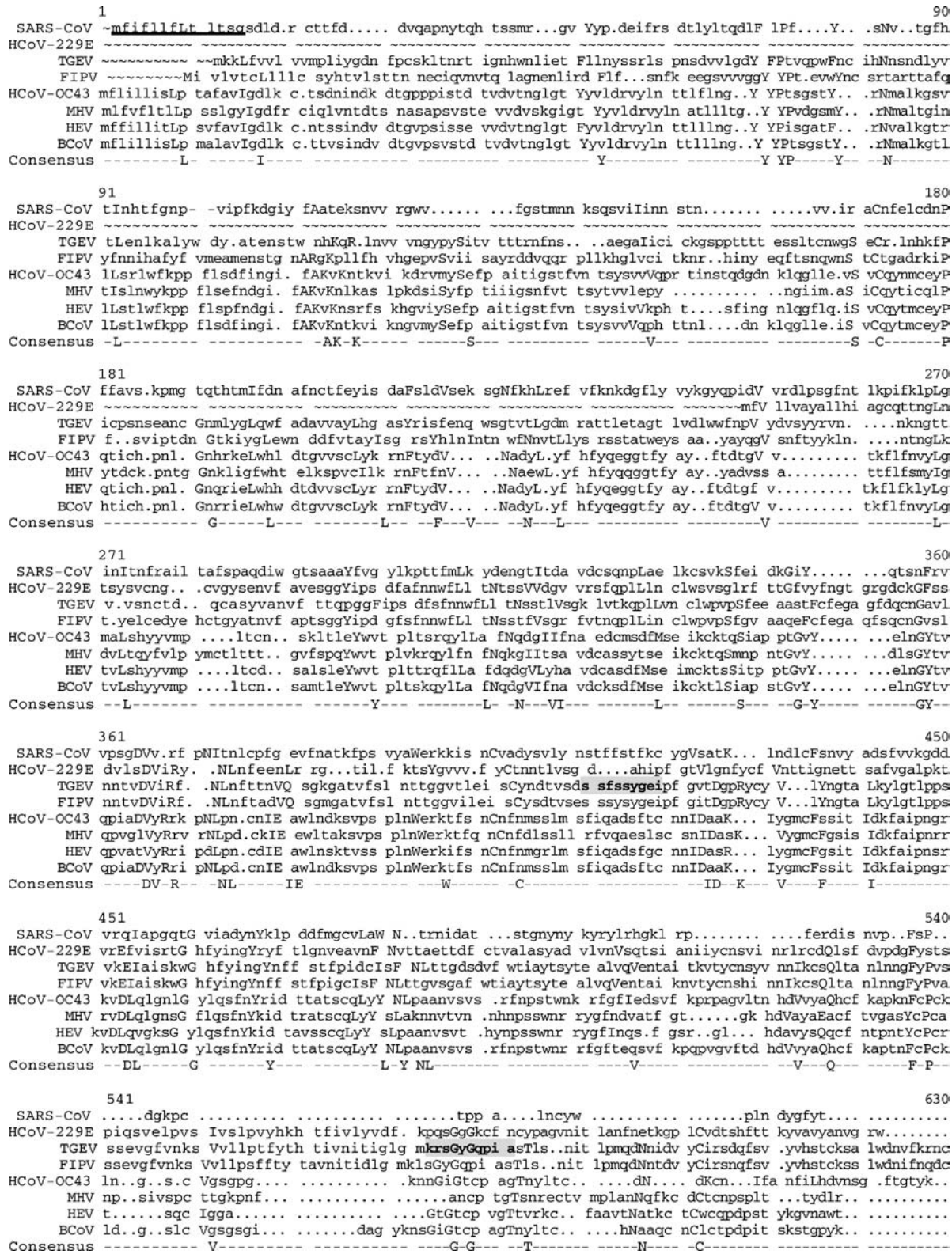

Fig. 2. Multiple alignment of coronavirus S proteins. Amino acid sequences of S proteins from human respiratory coronavirus (HCoV-229E) (GenBank Accession No. P15423), porcine transmissible gastroenteritis virus (TGEV) (GenBank Accession No. CAA37285), feline infectious peritonitis virus (FIPV) (GenBank 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, and 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.

```
          631                                                                                                  720
 SARS-CoV .........tt giG....... .......... .......... .......yqp yRvv...Vls felLna..pa tvcgpkl... stD...likn
HCoV-229E ......sasI ntGncpfsfg kvnnfvkfgs vCfslkdipg gcampivanw ayskyytIgs lyvswsdgdg itgvpqpveg vssfmnvtld
     TGEV tdvldatavI ktGtcpfsfd klnnyltfnk fClslspvga nckfDvaar. tRandqvVrs lyvIyeegdn ivgvpsdnsg lhDlsvlhld
     FIPV tdvlEatavI ktGtcpfsfd klnnyltfnk fClslspvga nckfDvaar. tRtneqvVrs lyvIyeegdn ivgvpsdnsg lhDlsvlhld
HCoV-OC43 ..cpQtkslV giGehcsgla vksdycgg.n sCtcrpqafl gwsaDsclqg ltcstdlqka ntD...iilg vCvnYdLYGI
      MHV ..clQarsmL gvGdhceglg vledkcggsn tCncsahafv gwakDsclan gRch...Ifs nlmLnginsg ttcsmdlqlp ntE...vvtg
      HEV ..cpQskvsI qpGqhcpglg lveddcsg.n pCtckpqafi gwssEtclqn gRcn...Ifa nfiLndvnsg ttcstdlqqg ntn...ittd
     BCoV ..cpQtkylV giGehcsgla iksdycgg.n pCtcqpqafl gwsvDsclqg dRcn...Ifa nfiLhdvnsg ttcstdlqks ntD...iilg
Consensus ----Q----I --G------- ---------- -C-------- ----D----- -R-----I-- ---L------ ---------- --D-------

          721                                                                                                  800
 SARS-CoV qCvnFnfnGL tGtGVltp.s skrfqpfqqf grdv.sdftd svRDpktsei ldIspCsfgg vsvitpgtna ssevavlYqd vnctdVstai
HCoV-229E kCtkYnIYdV sGvGVirvsN dTfln...gI tYtStsGnLl GFKDvtkgti YsItpCnppd qlvvyqqavv gamlsenFts ygfsnVve..
     TGEV sCtdYnIYGr sGvGIirqtN rTlls...gL yYtSlsGdLl GFKnvsdgvi YsVtpCdvsa qaAvidgtiv gaitsInsel Lglthwtt..
     FIPV sCtdYnIYGr tGvGIirrtN sTlls...gL yYtSlsGdLl GFKnvsdgvi YsVtpCdvsa qaAvidgaiv gamtsInsel Lglthwtt..
HCoV-OC43 lGqGIfvevN aTyynswqnL lYdS.nGnLy GFRDyiinrt FmIrsCysgr vsAafhan.. ssepaLlFrn IkcnyVfnns ltrqlqPi..
      MHV vCvkYdLYGI tGqGIfkevk adyyhswqnL lYdv.nGnLi GFRDfvanks YtIrsCysgr vsAayhqd.. apepaLlYrn LkcdyVfnnn
      HEV vCvnYdLYGI tGqGIlievN aTyynswqnL lYdS.sGnLy GFRDylsnrt FlIrsCysgr vsAvfhan.. ssepaLmFrn LkcshVfnyt
     BCoV vCvnYdLYGI tGqGIfvevN aTyynswqnL lYdS.nGnLy GFRDyltnrt FmIrsCysgr vsAafhan.. ssepaLlFrn IkcnyVfnnt
Consensus -C--Y-LYGI -G-GI----N -T-------L -Y-S--G-L- GFRD------ Y-I--C---- --A------- -----L-F-- L----V----

          801                                                                                                  890
 SARS-CoV hadqltPawr iystgnnvFq tqaGclIgae hvdts..yec dipigaGiCa syhtvslIr. ..stsqk..s ivaytmslga DSsiaysn.n
HCoV-229E  .....lPkff yas..ngtYn ct........ ....daVlTy s...sfGvCa dgsiiavq.. .......... ....prnvsy DSVsaIv.ta
     TGEV .....tPnfy yysiynytnD mtrGtaIdsn dvdcepViTy s...niGvCk ngalvfin.. .......... ....vthsdg D.VQpIs.tg
     FIPV .....tPnfy yysiynytsE rtrGtaIdsn dvdcepViTy s...niGvCk ngalvfin.. .......... ....vthsdg D.VQpIs.tg
HCoV-OC43 ......nyFD sylGcvVnay nstaisVqTc dltvgsGyCv dysknrrsrg aittgyrftn fepftvnsvn DSlEpVggly eIqIPseFTI
      MHV isreetPl.. ......nyFD sylGcvVnad nsteeaVdac dlrmgsGlCv nystshrars svstgykltt fepftvrivn DSVEsVdgly
      HEV ilrqiqlv.. ......nyFD sylGcvVnay nntasaVsTc dltvgsGyCv dyvtalrsrr sfttgyrftn fepfaanlvn DSIEpVggly
     BCoV lsrqlqPi.. ......nyFD sylGcvVnad nstssaVqTc dltvgsGyCv dystkrrsrr aittgyrftn fepftvnsvn DSlEpVggly
Consensus ------P--- --------FD ---G--V--- ------V-T- ------G-C- ---------- ---------- ---------- DSVE-V----

          891                                                                                                  980
 SARS-CoV tIaIPtnFsI sittEvmpvs maktsVDCnm yICgdsteCa nlLlqYgSfC tqLnraLsgi aaeq[drntr]E vfaqvkqmyk tptlkyfG..
HCoV-229E  nLsIPsnwTt svqvEYLQit stpIvVDCst yVCngnvrCv elLkqYtSaC ktIedaLrns arLesadvsE mltfdkkaft LanvssfGd.
     TGEV nVtIPtnFTI svqvEYIQvy ttpVsIDCsr yVCngnprCn klLtqYvSaC qtIeqaLavg arLenmevds mlfvsenalk Lasveafnss
     FIPV nVtIPtnFTI svqvEYmQvy ttpVsIDCar yVCngnprCn klLtqYvSaC qtIeqaLamg arLenmevds mlfvsenalk Lasveafnst
HCoV-OC43 gnmvEFIQts spkVtIDCaa fVCgdyaaCk sqLveYgSfC DnInaiLtev neLldttqlQ vanslmngvt LstklkdGvn fnvDdI..nf
      MHV eLqIPtnFTI ashqEFVQtr spkVtIDCaa fVCgghtaCr qqLveYgSfC DnInaiLgev nnLidtmqlQ vasaliqgvt LssrlsdGig
      HEV eIqIPseFTI gnleEFIQts spkVtIDCat fVCgdyaaCr qqLaeYgSfC EnInaiLiev neLldttqlQ vanslmngvt LstkikdGin
     BCoV eIqIPseFTI gnmeEFIQis spkVtIDCsa fVCgdyaaCk sqLveYgSfC DnInaiLtev neLldttqlQ vanslmngvt LstklkdGvn
Consensus -I-IP--FTI ----EFIQ-- ---V-IDC-- -VC-----C- --L--Y-S-C D-I---L--- --L------Q ---------- L------G--

          981                                                                                                 1070
 SARS-CoV ....gf..nf sqil...... ...pdplkpt k...RSfIED LLFnKVtlad aGfmkq.Yge ClGdinarDL ICaQkFNGLt VLPplltddm
HCoV-229E ynlssVi... ps........ ....lptsgS rvagRSAIED ILFsKlvtSg lGtVdadYkk CTkGlsIaDL aCaQyYNGIm VLPgvadaer
     TGEV etlDpIykew pnigGswleg lkyilpsdnS krkyRSAIED LLFsKVvtSg lGtVdedYkr CTGGyDIaDL VCaQyYNGIm VLPgvanadk
     FIPV enlDpIykew psigGswlgg lkdilpshnS krkygSAIED LLFdKVvtSg lGtVdedYkr CTGGyDIaDL VCaQyYNGIm VLPgvanadk
HCoV-OC43 spvlGclgse cska.....S s...RSAIED LLFdKVklSd vGfVea.Ynn CTGGaEIrDL ICvQsYkGIk VLPpllsenq isgYTlaats
      MHV gqiDdI..nf spllGclgsd cgevtmaaqt g...RSAIED vLFdKVklSd vGfVea.Ynn CTGGqEVrDL LCvQsFNGIk VLPpvlsenq
      HEV fnvDdI..nf ssvlGclgse cnra.....S t...RSAIED LLFdKVklSd vGfVqa.Ynn CTGGaEIrDL ICvQsYNGIk VLPpllsenq
     BCoV fnvDdI..nf spvlGclgsd cnkv.....S s...RSAIED LLFsKVklSd vGfVea.Ynn CTGGaEIrDL ICvQsYNGIk VLPpllsenq
Consensus ---D-I---- ----G----- ----------S ----RSAIED LLF-KV--S- -G-V---Y-- CTGG-EI-DL IC-Q-YNGI- VLP-------

          1071                                                                                                1160
 SARS-CoV iaaYTaalvs gtatagwtfg aGaalqiPFa MqmayRfNgi gvtqnVLyeN QKqIAnqFNr AisqIqEslt tts....... .......tAL
HCoV-229E mamYTgslig gial...ggl .tsavsiPFs LaIQaRLNyv alqtDVLqeN QKiLAasFNk AmtnIvDaFt gvNdaitqts qalqtvatAL
     TGEV mtmYTaslag gitl...gAl gGgavaiPFa vaVQaRLNyv alqtDVLnkN QqiLAsAFNq AignItQsFg kvNdaihqts rglatvakAL
     FIPV mtmYTaslag gitl...gAl gGgavaiPFa vaVQaRLNyv alqtDVLnkN QqiLAnAFNq AignItQaFg kvNdaihqts qglatvakAL
HCoV-OC43 aslfppwtAa aG....vPFy LnVQyRINgl gvtmDVLsqN QKlIAnAFNn AlyaIqEgFd atN....... .......sAL vKIQaVVNan
      MHV isgYTagatv samf.pwsAa aG....vPFs LsVQyRINgl gvtmnVLseN QKmIAsAFNn AigaIqEgFa atN....... .......sAL
      HEV isgYTsaata aslfppwtAa aG....vPFy LnVQyRINgl gvtmDVLsqN QKlIAsAFNn AldsIqEgFd atN....... .......sAL
     BCoV isgYTlaats aslfppwsAa aG....vPFy LnVQyRINgi gvtmDVLsqN QKlIAnAFNn AldaIqEgFd atN....... .......sAL
Consensus ---YT----- --------A- -G-----PF- L-VQ-RIN-- ----DVL--N QK-IA-AFN- AI--I-E-F- --N------- --------AL


          1161                                                                                                1250
 SARS-CoV gKLQdVVNqn aQALNtLvkQ LssnFgAISs vLnDIlsRLD kvEAEvQIDR LItGRLqsLq tYVtQqLira aeIraSanlA atKmsECVlg
HCoV-229E nKIQdVVNqq gnsLNhLtsQ LrqnFqAISs SIqaIydRLD tIQADqQVDR LItGRLaALN vFVShtLtky teVraSrqlA qQKVNECVKS
     TGEV aKVQdVVNtq gQALshLtvQ LqNnFqAISs SIsDIynRLD eLsADAhVDR LItGRLTALN AFVSQtLtrq aeVraSrqlA kDKVNECVRS
     FIPV aKVQdVVNtq gQALshLtvQ LqNnFqAISs SIsDIynRLD eLsADAQVDR LItGRLTALN AFVSQtLtrq aeVraSrqlA kDKVNECVRS
HCoV-OC43 aEALNnLlqQ LsNrFgAISa SLqEIlsRLD aLEAEAQIDR LInGRLTALN AYVSQqLsds tlVkfSaaqA mEKVNECVKS
      MHV aKmQfVVNan aEALNnLlnQ LsNrFgAISa SLqEIlsRLD aLEAqAQIDR LInGRLTALN AYVSkqLsdm tlVkvSaaqA iEKVNECVKS
      HEV vKIQaVVNan aEALNnLlqQ LsNrFgAISa SLqEIlsRLD aLEAkAQIDR LInGRLTALN AYVSQqLsds tlVkfSaaqA iEKVNECVKS
     BCoV vKIQaVVNan aEALNnLlqQ LsNrFgAISs SLqEIlsRLD aLEAqAQIDR LInGRLTALN AYVSQqLsds tlVkfSaaqA mEKVNECVKS
Consensus -KIQ-VVN-- -EALN-L--Q L-N-F-AIS- SL-EI--RLD -LEADAQIDR LI-GRLTALN AYVSQ-L--- --V--S---A -EKVNECVKS
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Fig. 2. (*continued*)

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          1251                                                                                                1340
 SARS-CoV QSkRvdFCGk GyHLmSfpqa APhGvvFLHv tyvPsqernf ttapaIChe. GE...ayfpR e...gvFvfn gtsWfiTqrn fFsPqiiTtd
HCoV-229E QSkRygFCGN GtHIfSIvna APeGLvFLHt vllPTqYkdv eawsGLCv.. .DgtnGyvlR qpnlalYke. gnyYriTsri mFePripTma
     TGEV QSqRfgFCGN GtHLfSLana APnGMiFfHa vllPTaYeTv tawaGICald GDrtfGlvvK dvqltlFrnl ddkfylTprt mYqPrvaTss
     FIPV QSqRfgFCGN GtHLfSLana APnGMiFfHt vllPTaYeTv tawsGICasd GDrtfGlvvK dvqltlFrnl ddkfylTprt mYqPrvaTss
HCoV-OC43 QSsRinFCGN GnHIiSLvqn APyGLyFIHf syvPTkYvTa rvspGLCia. GDr..GiapK s...gyFvnv nntWmyTgsg yYyPepiTen
      MHV QSsRinFCGN GnHIlSLvqn APyGLyFIHf syvPTsFtTa nvspGLCis. GDr..GlapK a...gyFvqd dgeWkfTgsn yYyPepiTdk
      HEV QSsRinFCGN GnHIiSLvqn APyGLyFIHf syvPTkYvTa kvspGLCia. GDi..GispK s...gyFinv nnsWmfTgsg yYyPepiTqn
     BCoV QSsRinFCGN GnHIiSLvqn APyGLyFIHf syvPTkYvTa kvspGLCia. GDr..GiapK s...gyFvnv nntWmfTgsg yYyPepiTgn
Consensus QS-R--FCGN G-HI-SL--- AP-GL-FIH- ---PT-Y-T- ----GLC--- GD---G---K ------F--- ---W--T--- -Y-P---T--

          1341                                                                                                1430
 SARS-CoV ntfvsgnCdV vigiinntvy dplqPeldsf k...EELdky FKnhts..PD vdlgdisgiN asvvnIqkEi d..... .....RLsev
HCoV-229E dfVqienCnV tFvnisrseL qtivPeyiDv nktlQEL..s YKlpnytvPD LvveqY...N qTiLnLtsEi stlenksael nytvqKLqtl
     TGEV dfVqiegCdV lFvnAtlsdL psiiPdyiDi nqtvQDIlen FR.pnwtvPE LtfdiF...N aTyLnLtgEi ddlefrsekl hnttveLail
     FIPV dfVqiegCdV lFvnAtvidL psiiPdyiDi nqtvQDIlen YR.pnwtvPE ftldiF...N aTyLnLtgEi ddlefrsekl hnttveLail
HCoV-OC43 nvVvmstCaV nYtkApyvmL ntsiPnlpDf k...EELdqw FKnqtsvaPD Lsl.dY..iN vTfLdLqvEm n..... .....RLqea
      MHV nsVvmssCaa nYtkApevfL ntsiPnlpDf k...EELdkw FKnqtsiaPD Lsl.dFeklN vTlLdLtdEm n..... .....RIqda
      HEV nvVvmstCaV nYtkApdlmL ntstPnlpDf k...EELyqw FKnqsslaPD Lsf.dY..iN vTfLdLqdEm n..... .....RLqea
     BCoV nvVvmstCaV nYtkApdvmL nistPnlpDf k...EELdqw FKnqtsvaPD Lsl.dY..iN vTfLdLqdEm n..... .....RLqea
Consensus --V----C-V -Y--A----L ----P---D- ----EEL--- FK------PD L----Y---N -T-L-L--E- ---------- -----RL---

          1431                                                                                                1526
 SARS-CoV aknLNeslId LqeLgkyEqY IKWPWYVWLg fiaglIaivm vtillCCmTs C.csclkgac sCgsCCkfde ddsepvLkgv klhyt~
HCoV-229E IdnINstlVd LkwLnrvEtY IKWPWWVWLc IsvvlIfvvs MLllcCCsTG Ccgffscfas sirgCCestk lpyyd.Veki hiq~~~
     TGEV IdnINntlVN LewLnriEtY VKWPWYVWLL IgLvvIfcIp LLlFcCCsTG Ccgcigclgs cChsiCsrrq fenyepIekv hih~~~
     FIPV IdnINntlVN LewLnriEtY VKWPWYVWLL IgLvvVfcIp LLlFcCfsTG Ccgcigclgs cChsiCsrrq fenyepIekv hvh~~~
HCoV-OC43 IkvLNqsyIN LkdIgtyEyY VKWPWYVWLL IcLagVamLv LLfFiCCcTG Cgtscfk...kCggCCddyt gyqelvI... ktshdd
      MHV IkkLNesyIN LkdvgtyEmY VKWPWYVWLL IgLagVavcv LLfFiCCcTG Cgsccfk...kCgnCCdecg ghqdsiVihn isshed
      HEV IkvLNhsyIN LkdIgtyEyY VKWPWYVWLL IcLagVvmLv LLfFiCCcTG Cgtscfk...kCggCfddyt ghqefvI... ktshdd
     BCoV IkvLNqsyIN LkdIgtyEyY VKWPWYVWLL IgLagVamLv LLfFiCCcTG Cgtscfk...kCggCCddyt ghqelvI... ktshdd
Consensus I--LN---IN L--L---E-Y VKWPWYVWLL I-L--V--L- LL-F-CC-TG C----------C--CC---- ------I--- ------
```

Fig. 2. (*continued*)

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.

For biotinylated Western blot, cell extracts were separated by 10% SDS–PAGE and the protein bands were then transferred to membrane. After blocking, the membrane was probed with 0.1 μg/ml biotinylated S protein at 4 °C for 1 h. The bound protein was detected with peroxidase-conjugated avidin (Sigma) followed by chemiluminescence and exposed by X-ray films.

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. After three washes with washing buffer, 50 μl diluted peroxidase-conjugated secondary antibody was added to each well and incubated at 37 °C for 1 h. Following three washes, 50 μl chromogenic substrate, 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid), 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.

For biotinylated ELISA, the procedure was performed as described previously with modification. Briefly, microtiter plates were coated with 5 μg/well cell extract which was diluted in 0.05 M carbonate buffer, blocked with blocking buffer, and challenged with 0.1 μg/well biotinylated S protein. After a 1-h incubation at 37 °C, diluted peroxidase-conjugated avidin was added 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. S gene had an ORF of 3768 nucleotides, capable of coding for a 1255-amino-acid polypeptide of about 138 kDa. The amino terminus of SARS-CoV S protein contained a short type I signal peptide (residue 1–13) composed of hydrophobic amino acids that are presumably removed during cotranslational transport through ER. The carboxyl terminus (residue 1196–1255) consisted of a transmembrane

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

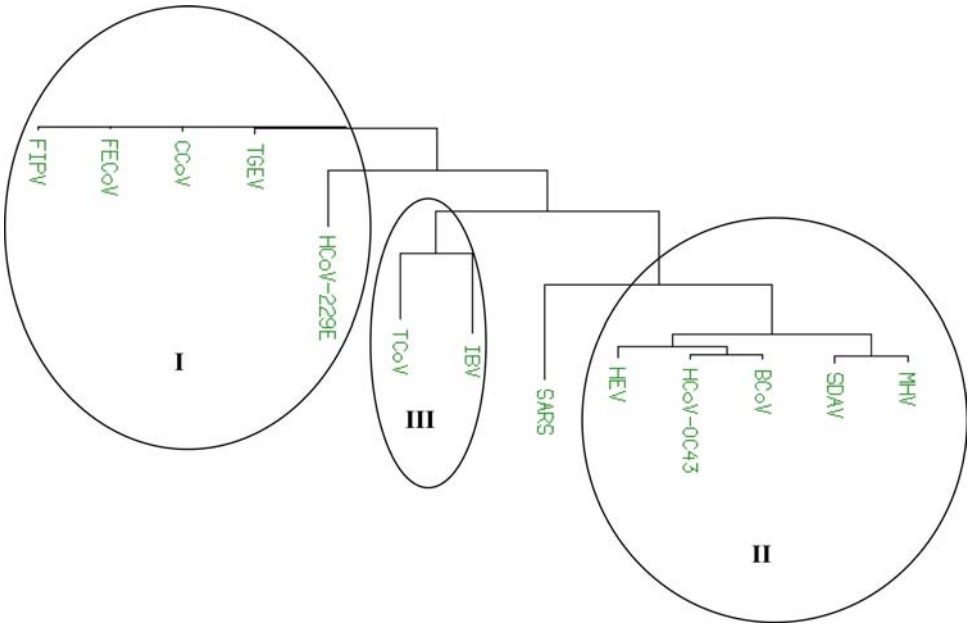

Fig. 3. Phylogenetic analysis of coronavirus S proteins. Amino acid sequences of SARS-CoV S proteins were compared with those of HCoV-229E, TGEV, FIPV, HCoV-OC43, MHV, HEV, BCoV, avian infectious bronchitis virus (IBV) (GenBank Accession No. P11223), and turkey coronavirus (TCoV) (GenBank Accession No. AAQ20922). Sequence alignment and neighbor-joining tree were generated by GrowTree program of GCG using the BLOSUM comparison matrix. Coronavirus groups I, II, and III are indicated. Branch lengths are proportionate to amino acid differences.

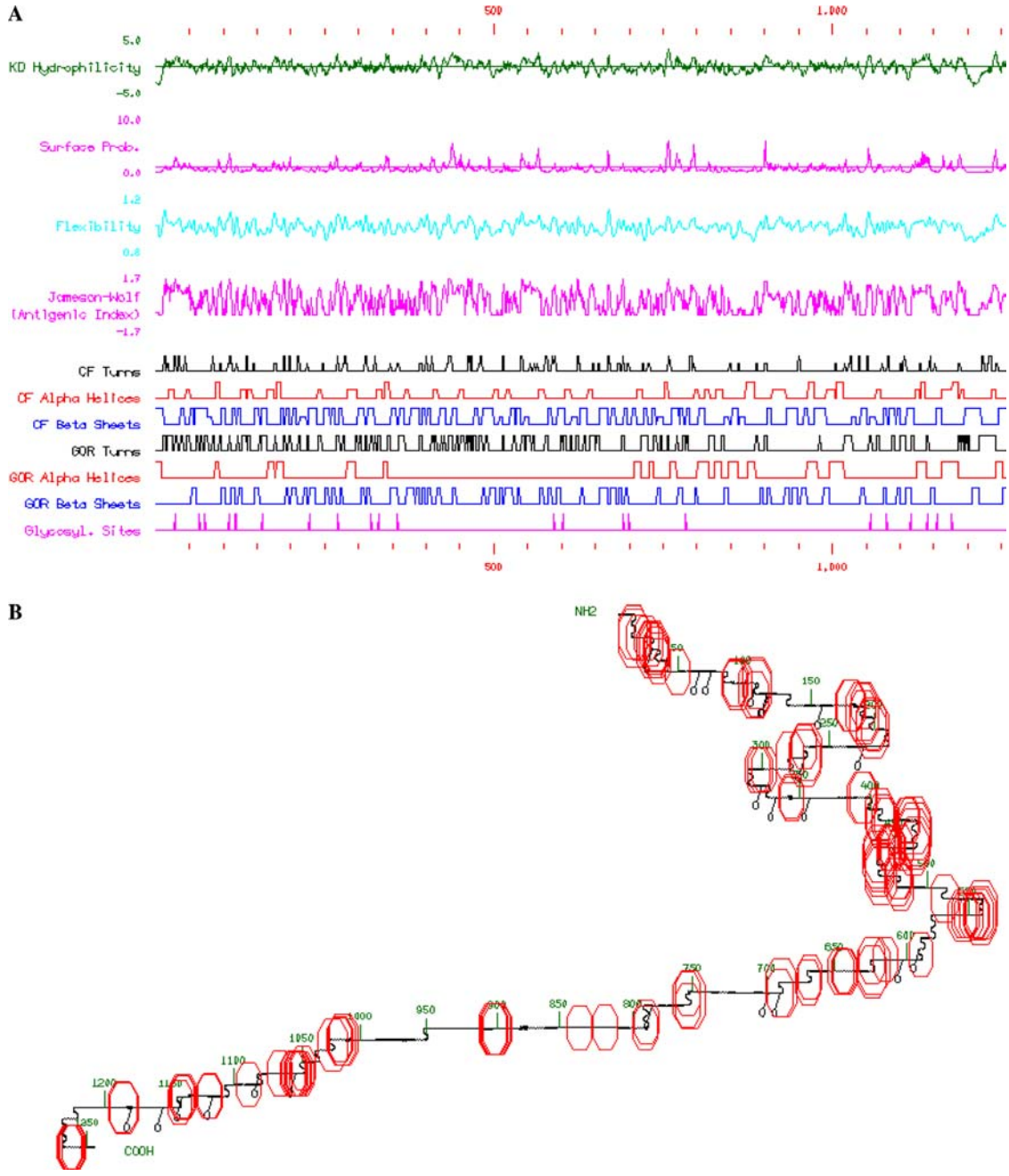

Fig. 4. Analysis of the primary sequence of SARS-CoV S protein. (A) The hydrophilicity, surface probability, and chain flexibility of SARS-CoV S protein were analyzed by PeptideStructure program of GCG. (B) Antigenic analysis of SARS-CoV S protein. The secondary structure and putative antigenic sites of SARS-CoV S protein were analyzed by PeptideStructure program of GCG using the Chou-Fasman prediction. Secondary structure was drawn with the PloStructure program. The predicted antigenic sites are shown as octagons. The putative glycosylation sites are shown as balloons.

analysis (Fig. 5). 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

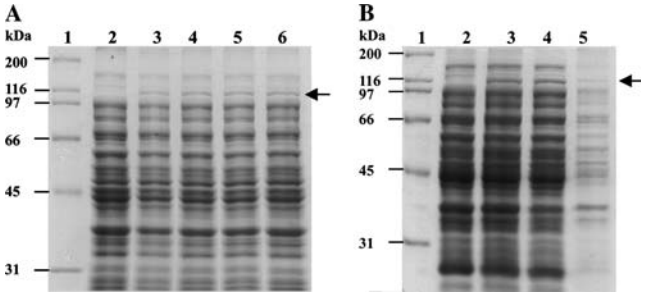

Fig. 5. Optimal condition and distribution of recombinant SARS-CoV S protein in *E. coli*. (A) Optimal condition for expressing recombinant S protein. The *E. coli* BL21(DE3)pLysS strain containing pET-spike was inoculated at 37 °C with shaking. After an overnight incubation, the bacteria were refreshed to 0.45 (lane 3), 0.5 (lane 4), 0.55 (lane 5) or 0.6 OD_{600} (lane 6) and the IPTG was then added to induce the protein expression. The preparations of uninduced (lane 2) and induced *E. coli* (lanes 3–6) were analyzed by 10% SDS–PAGE and stained by Coomassie brilliant blue. (B) Distribution of recombinant S protein in *E. coli*. After the induction of IPTG, the total proteins (lane 3), soluble (lane 4), and insoluble fractions (lane 5) of induced *E. coli* were analyzed by 10% SDS–PAGE and stained by Coomassie brilliant blue. Lane 2 represents the preparation of uninduced *E. coli*. The molecular masses of protein standard (lane 1) are shown at the left. The 138-kDa recombinant S protein is indicated by the arrow.

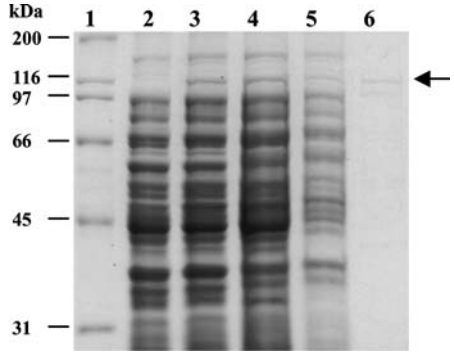

Fig. 6. SDS–PAGE analysis of recombinant SARS-CoV S protein. The total proteins (lane 3), soluble (lane 4), unbinding (lane 5), and purified fractions (lane 6) of induced *E. coli* were analyzed by 10% SDS–PAGE and stained by Coomassie brilliant blue. Lane 2 represents the preparation of uninduced *E. coli*. The molecular masses of protein standard (lane 1) are shown at the left. The 138-kDa recombinant S protein is indicated by the arrow.

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

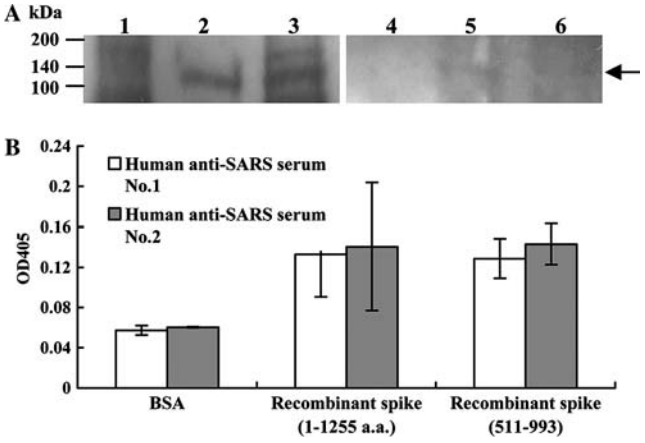

Fig. 7. Antigenic analysis of recombinant SARS-CoV S protein. (A) Western blot analysis. The preparations of uninduced *E. coli* (lanes 1 and 4), induced *E. coli* (lanes 3 and 6), and purified S protein (lanes 2 and 5) were analyzed by 10% SDS–PAGE, transferred to nitrocellulose membrane, detected by rabbit anti-spike antibody (lanes 1–3) or human anti-SARS-CoV antibody (lanes 4–6), and subsequently visualized by chemiluminescence. (B) ELISA. The BSA and recombinant S proteins (residue 1–1255 and residue 511–993) were coated on ELISA plates and detected by human anti-SARS-CoV antibody. Values are means ± standard error of triplicate assays.

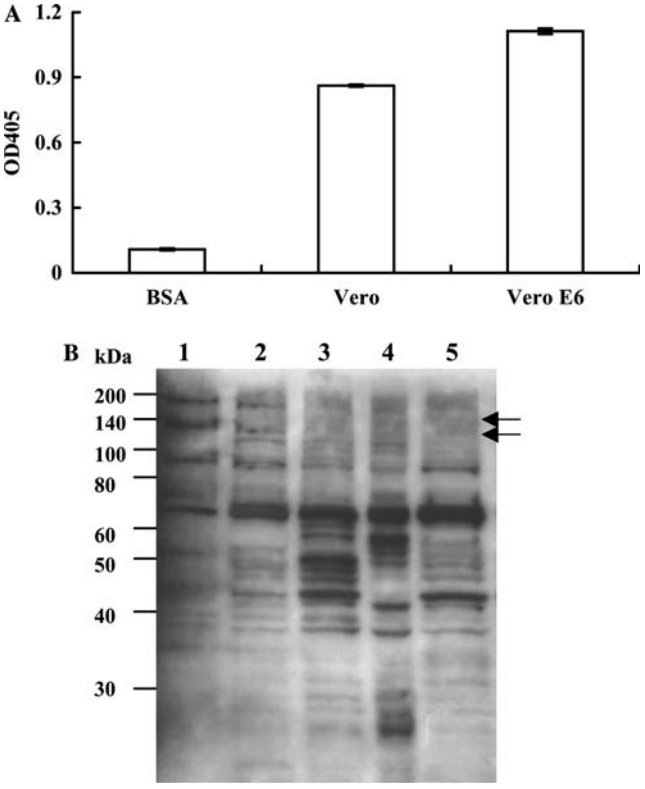

Fig. 8. Receptor-binding ability of recombinant S protein. (A) Biotinylated ELISA. The BSA, Vero cell extract, and Vero E6 cell extract were coated on ELISA plates and detected by biotin-labeled S protein. Values are means ± standard error with triplicate assays. (B) Biotinylated Western blot. The cell extracts of Vero E6 (lane 1), Vero (lane 2), A-549 (lane 3), Chang liver (lane 4), and U-937 (lane 5) were separated by SDS–PAGE, transferred to membrane, and detected by biotin-labeled S protein. The molecular masses of protein standard are shown at the left. The proteins present in Vero cells only are indicated by arrowheads.

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.

Acknowledgments

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