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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.

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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'-GGAATTCCGCAACATACCAAGGCC-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 PfuUltra DNA polymerase (Stratagene) with SARS-P1 primer (5'-CGGGATCCTAGTGGTAGTGACCTTGACC-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 *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 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'-TTGATATAGAACAGCAACTTCAGATGAAGC-3'), or SARS-M1 (5'-GGAATTCCACTTACACCCCAAAAGAGC-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 OD₆₀₀ 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 Na₂HPO₄, and 1.4 mM KH₂PO₄) 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% CO₂ 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.

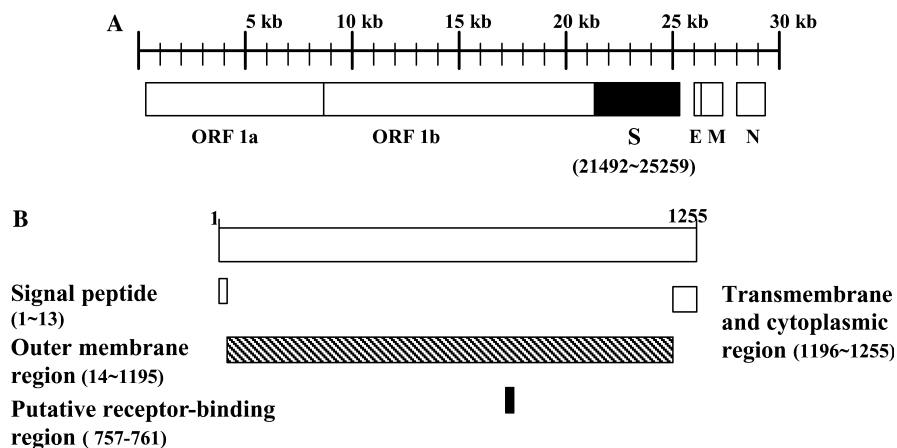


Fig. 1. Genome organization of SARS-CoV and putative region of SARS-CoV S protein. (A) Overall organization of the 29,727-nt SARS-CoV. Predicted ORF 1a and ORF 1b encode replicase 1A and 1B, respectively. S, E, M, and N genes encode spike, envelope, membrane, and nucleocapsid proteins, respectively.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

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1
SARS-CoV mfifllflftl lrsqsdld.r cttfd.... dvqapnytqh tssmr...gv Yyp.deifrs dtlyltqdlF lPf...Y. .sNv..tgfh
HCoV-229E -----
TGEV ----- ~mkLfvvl vvmpliygdn fpcsklnrt ignhwnliet Fllyssrsls pnsdvvlgdY FPtvpqvfnc ihNnsndlyv
FIPV ----- Mi vlvtcLlllc syhtvlsttn neciqvmtq lagenliard Flf...snfk eegsvvvggY YPt.ewwYnc strartatfq
HCoV-OC43 mflilllisLp tafavIgdIk c.tsdnindk dtgpppistd tvdvtnglgt Yyvldrlyln ttlling..Y YPtsgstY.. rNmalkgsv
MHV mlfvfltLlp sslgyIgdfr ciqlvntdts nasapsvste vvdvskgigt Yyvldrlyln atllltg..Y YPvdgsmY.. rNmaltgin
HEV mffillitLp svfavIgdIk c.ntssindv dtgvpsisse vvdvtnglgt Fyvldrlyln ttlling..Y YPisgatF.. rNvalkgtr
BCoV mflilllisLp malavIgdIk c.ttvsvindv dtgvpsvstd tvdvtnglgt Yyvldrlyln ttlling..Y YPtsgstY.. rNmalkgtl
Consensus -----L-----I-----Y-----Y YP-----Y-----N-----

91
SARS-CoV tInhtfgnp- vipfkdgiy fAateksnv rgwv..... ..fgstmmn ksqsviInn stn..... ..vv.ir aCnfcldnP
HCoV-229E -----
TGEV tLenlkalyw dy.atenstw nhKqR.lnvv vngypySiv tttrmfns.. .aegaIici ckgspptttt esslctnwgS eCr.lnhkfP
FIPV yfnnihafyf vmeamenstg nARgKpIlfh vhegvsVii sayrddvqgr pllkhlvci tknr..hiny eqftsnqwmS tCtgadrkiP
HCoV-OC43 lLsrLwfKpp flsdfingi. fAKvKntkvi kdrvmYsefp aitagstfvn tsysvVqpr tinstqdgdn klqgll.e.vS vCYmnceyP
MHV tIsinwykpp flsefndgi. fAKvKnlkas lpkdsiSyfp tiiigsnfvt tsyvvlepy ..... .ngiim.as iCqyticlP
HEV lLstlwfKpp flspfdngi. fAKvKnsrfs khgviySefp aitagstfvn tsysivVkpH t....sfing nlqgflq.iS vCYtmceyP
BCoV lLstlwfKpp flsdfingi. fAKvKntkvi kngvmySefp aitagstfvn tsysvVqph ttnl...dn klqgll.e.iS vCYtmceyP
Consensus -L-----AK-K-----S-----V-----S-----C-----P

181
SARS-CoV ffavs.kpmg tqthtmIfdn afnctfeyis daFslDvsek sgNfkhLref vfkndkgfly vykgyqpidV vrdlpqgnt lkpifklpLg
HCoV-229E -----
TGEV icpsnseanc GnmlyLqwf adavayLhg asYrisfeng wsgvtLgdm rattletagt lvdllwfnpV ydvsyvrln. ....nkngtL
FIPV f..sviptdn GtkiygLeWn ddfvtayIsg rsYhlnIntn wfnmvtLlys rsstatweys aa..yayqgV snftyykl. ....ntngLk
HCoV-OC43 qtich.pnl. GnhrikeLwhl dtgvvscLyk rnFtydV... ..NadyL.yf hfyqeggtfy ay..ftdtgV v..... tkflfnvyLg
MHV ytdck.pntg GnkligfwhT elkspvcIlk rnFtnV... ..NaewL.yf hfyqeggtfy ay..yadvss a..... ttflfsmYg
HEV qtich.pnl. GnqrieLwhh dtdvvsLyr rnFtydV... ..NadyL.yf hfyqeggtfy ay..ftdtgV v..... tkflfkyLg
BCoV htich.pnl. GnrrieLwhw dtgvvscLyk rnFtydV... ..NadyL.yf hfyqeggtfy ay..ftdtgV v..... tkflfnvyLg
Consensus -----G-----L-----L-----F-----V-----N-----L-----V-----L-----

271
SARS-CoV inItnfrail tafspaQdiw gtsaaaYfvg ylkpttflmLk ydengtItDa vdcsgmpLae lkcsvkSfei dkGiY.... ..qtsnFrV
HCoV-229E tsysvcng.. .cvgysevf avesggYips dfafnnwFl tNtssVVdgV vrsfqplLln clwsvsglrf ttGfvyngt grgdckGFss
TGEV v.vsnctd.. qcasvyanvf ttqpggFips dffsnmWfl tNsstlVsgk lvtkqplLvn clwvpsfFee aastFcfega gfdqcnGavl
FIPV t.yelcedye hctgyatnvf aptsggYipd gfsfnmWfl tNsstfVsgR fvtngqplLin clwvpsfGv aaqeFcfega qfsqcnGvsl
HCoV-OC43 maLshyyvmp ....ltn.. skltleYwvt pltsrqyLLa fNqdgIIfna edcmsdfMse ikcktqSiap ptGvY.... ..elnGytv
MHV dvLqtqYvlp ymctlttt.. gvfsppYwvt plvkrqyfln fNqgKIItsa vdcassytse ikcktqSmpn ntGvY.... ..dlSGYtv
HEV tvLshyyvmp ....ltd.. salsleYwvt pltrqfLLa fdqdgVILya vdcasdfMse imcktsSitp ptGvY.... ..elnGytv
BCoV tvLshyyvmp ....ltn.. samtleYwvt pltsrqyLLa fNqdgVIFna vdcasdfMse ikcktlSiap stGvY.... ..elnGytv
Consensus --L-----Y-----L-----N-----VI-----L-----S-----G-Y-----GY--

361
SARS-CoV vpsgDVv.rf pNItnlcpfg evfnatkfps vyaWerkkis nCvadysvly nstffstfkc ygVsatK... lndlcFsnvy adsfvkvgdD
HCoV-229E dVlsDViRy.. .NLnfeenLr rg...til.f ktsYgvvV.f yCtnntlvsg d...ahipf gtVlmgfyf Vtignnet safvagalpKt
TGEV nntvDViRf.. .NLnfttnVQ sgkgatvfls nttggvtlei sCyndtvdsd sfssygeipf gvtDgpRycy V...lyngta Lkylgtlpps
FIPV nntvDViRf.. .NLnftadVQ sgmgatvfls nttggvilei sCysdvtvss ssysygeipf gtDgpRycy V...lyngta Lkylgtlpps
HCoV-OC43 qpiaDVyRrk pNLpn.cnIE awlndksvps plnWerktkfs nCnfmmsslm sfiqadsfct nnIDaK... IygmCFssit Idkfaipngr
MHV qpvgLVyRrv rNLpd.ckIE ewltaksvps plnWerktkf nCnfdlssl rfvqaeslsc snIDaK... VygmCFgsis Idkfaipnrr
HEV qpvatVyRri pDLpn.cdIE awlnsktvss plnWerktkfs nCnfmmgrlm sfiqadsfct nnIDaR... IygmCFssit Idkfaipnrr
BCoV qpiaDVyRri pNLpd.cnIE awlndksvps plnWerktkfs nCnfmmsslm sfiqadsfct nnIDaK... IygmCFssit Idkfaipngr
Consensus ----DV-R-- -NL-----IE -----W-----C-----ID-K-- V---F--- I-----

451
SARS-CoV vrqIapgtqtG viadyNklp ddfmgcvLaW N..trmidat ...stgnyny kyrylrhngkl rp..... ..ferdis nvp..FSp..
HCoV-229E vrEfvisrtG hfyingYryf tlgvveavnF Nvtaettdf ctvalasyad vlvnVsqtSi aniiycnsiv nrIredQlSf dvpdgFysts
TGEV vkEiaiskwG hfyingYnff stfpidcIsF NLttgdsdvf wtiaytsye alvqVentali tkvtycnsiv nIkcsQlta nlngnFYpvs
FIPV vkEiaiskwG hfyingYnff stfpigcIsF NLttgvsGaf wtiaytsye alvqVentali knvtycnshi nnIkcsQlta nlngnFYpva
HCoV-OC43 kvDLqLgnlg ylgfsnYrid ttatscQLyY NLpaanvsvs .rfnpstwnk rfgfIedsvf kprpagvltN hdVvyaQhcf kapknFcPck
MHV rvDLqLgnsg flqfsnYkid ttatscQLyY sLaknvtvn .nhpsswnr rygfndvatf gt.....gk hdVayaEacf tvrgaYcPca
HEV kvDLqLgnsg ylgfsnYkid tavsscQLyY sLpaanvsvt .hynpsswnr rygfInqs.f gsr..gl... hdavysQhcf ntpntYcPcr
BCoV kvDLqLgnlg ylgfsnYrid ttatscQLyY NLpaanvsvs .rfnpstwnr rfgfteqsvf kppqvgvftd hdVvyaQhcf kaptnFcPck
Consensus --DL-----G-----Y-----L-Y NL-----V-----Q-----F-P--

541
SARS-CoV .....dgkpc ..... tpp a....lncyw ..... .pln dygfyt.... .....
HCoV-229E piqsvelpvs IvslpvYhkh tfivlyvdf. kpgsGgKcf ncyagvnt lanfnetkpp lCvdtshftt kyvavyanvg rw.....
TGEV ssevgfvnks Vvllptfyth tivnitiglg mksrGyGapi asTls..nit lpmqNnidv yCirsdqfsv .yvhstcksa lwdnVfkrnc
FIPV ssevgfvnks Vvllpsffty tavnitidlg mksrGyGapi asTls..nit lpmqNntdv yCirsngfsv .yvhstckss lwdnifngdc
HCoV-OC43 ln..g..s.c Vgsgppg..... .knnGiGtcp agTnyltc.. ..dn... dKcn...Ifa nfiLhdvnsG .ftgtyk..
MHV np..sivspc ttgkpnf..... ..ncp tgTsnrectv mplanNqfkc dCtcmplst...tydlr...
HEV t.....sqc Igga..... .GtGtcp vgTvrkc.. faavtNatkc tCwcqdpst ykvnmawt..
BCoV ld..g..slc Vgsgsgl... ..dag yknsGiGtcp agTnyltc.. ..hNaaqc nClctpdpit skstgpyk...
Consensus -----V-----G-----T-----N-----C-----

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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-CoVtt giG.....ygp yRvv...Vls felLna.pa tvcgpkll... stD...likn
HCoV-229EsasI ntGncpfsfg kmvfvkfgs vCfslkdiPg gcampivanw ayskyytIgs lyvswsdgdg itgvppqpvge vssfmvntld
TGEV tdvldatavI ktGtccpfsfd klunnyltfnk fClslspvga nckfDvaar. tRAndqvVrs lyvIyeeagn ivgvpsdngs lhdLsvlhld
FIPV tdvlEatavI ktGtccpfsfd klunnyltfnk fClslspvga nckfDvaar. tRtneqvVrs lyvIyeeagn ivgvpsdngs lhdLsvlhld
HCoV-OC43 ..cpQtkslv giGehcsgla vksdyccg.n sCtcrpqafl gwsaDscLqg ltcstldlqka ntD...iilg vCvnYdLYGI
MHV ..clQarsmL gvGdhceglg vledkccgns tCncsahafv gwakDscLan gRCh...Ifs nlmLnginsg ttcsmldqlp ntE...vvtg
HEV ..cpQskvsi qpGqhcpglg lveddcsg.n pCtckpqafl gwssEtclqn gRcn...Ifa nfiLndvnsq ttcstldlqg ntn...ittd
BCoV ..cpQtkylv giGehcsgla iksdyccg.n pCtccpqafl gwsvDscLqg dRcn...Ifa nfiLhdvnsq ttcstldlqks ntD...iilg
Consensus ----Q----I --G----- -C----- -D----- -R-----I--- --L----- ----- --D-----

721 800
SARS-CoV qCvnFnfnGL tGtGVltp.s skrfqpfqf grdv.sdftd svRDpktsei ldIspCsfgg vsvitpgtna ssevavlyQd vncdVstai
HCoV-229E kCtkYnIYdV sGvGVirvsN dtfln...gI tYtStsGnLl GFKDvtkgti YsItPcnpd qlvvyqgavv gamlsenFts ygfsnVve..
TGEV sCtdYnIYGr sGvGIrqtN rTlls...gL yYtSlsGdLl GFKnvsdgvI YsVtpCdvsA qaAvidgtiv gaitInsel lglthwtt..
FIPV sCtdYnIYGr tGvGIrrtN sTlls...gL yYtSlsGdLl GFKnvsdgvI YsVtpCdvsA qaAvidgaiv gamtsInsel lglthwtt..
HCoV-OC43 lGqGIfvevN aTyynswgnL lYdS.nGnLy GFRDylnrt FmIrsCysgr vsAafhan.. ssepaLlFrn lKcnyVfnns ltrqlqPi..
MHV vCvnYdLYGI tGgGIlievN aTyynswgnL lYdV.nGnLi GFRDylnrt YtIrsCysgr vsAayhd.. apepaLlFrn lKcdyVfnns
HEV vCvnYdLYGI tGgGIlievN aTyynswgnL lYdS.nGnLy GFRDylnrt FlIrsCysgr vsAvfhan.. ssepaLlFrn lKcshVfnnt
BCoV vCvnYdLYGI tGgGIfvevN aTyynswgnL lYdS.nGnLy GFRDylnrt FmIrsCysgr vsAafhan.. ssepaLlFrn lKcnyVfnnt
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 iystgnvFq tqaGclIgae hvdtS.yec dipigaGiCa syhtvslr. .stsqk..s ivaytmslga DSsiaysN
HCoV-229ElPkff yas.ngtYn ct..... .daVlTy s...sfGvCa dgsiiavq.prnvsvy DSVsaIv.ta
TGEVtPnfy yysiynyntD mtrGtaIdsn dvDcepViTy s...niGvCk ngalvfin.vthsdg D.VQpIs.tg
FIPVtPnfy yysiynyntE rtrGtaIdsn dvDcepViTy s...niGvCk ngalvfin.vthsdg D.VQpIs.tg
HCoV-OC43nyFD sylGcvVnay nstaisVqTc dvtGvYdCv dysknrrsrg aittgyrftn fepftvnsn DSlEpVggly eIqIpeFTI
MHV isreetPl..nyFD sylGcvVnad nsteevGdc dlrmgsGlcv nystshrrars svstgykltt fepftvtrivn DSVEsvdgly
HEV ilrqiglv..nyFD sylGcvVnay nntasaVsTc dltvgsGyCv dyvtalrsrr sfttgyrftn fepfaanlvn DSIEpVggly
BCoV lsrqlqPi..nyFD sylGcvVnad nstssaVqTc dltvgsGyCv dystknrrsrr aittgyrftn fepftvnsn DSlEpVggly
Consensus -----P--- ---FD ---G--V-----V-T-----G-C-----DSVE-V---

891 980
SARS-CoV tIaIPtnFsi sittEvmpvs maktsVDCnm yICgdsteCa nllLqYgSfC tqLnraLsgI aaeqdmrE vfaqvkmqk tptlkyfG..
HCoV-229E nLsIPsnwTt svqvEYLQit stpIvVDCst yVCnvnrcv eLlKqYtSaC ktIedaLrns arLesadvse mltfdkkaft LanvssfGd.
TGEV nVtIptnFTI svqvEYIqvy ttpVsIDCsr yVCnvnrcn klltQyVsaC qtIegaLavq arLenmevds mlfvsenalk Lasveafnss
FIPV nVtIptnFTI svqvEYmQvy ttpVsIDCar yVCnvnrcn klltQyVsaC qtIegaLavq arLenmevds mlfvsenalk Lasveafnss
HCoV-OC43 gnmvEFIQts spkVtIDCaa fVcGdyaaCq sqLveYgSfC DnInaiLtev neLldttqlQ vanslmngvt LstklkdGvn fnvDdI..nf
MHV eIqIptnFTI ashqEFVQtr spkVtIDCaa fVcGghtaCr qqLveYgSfC DnInaiLgev nnLidtmqlQ vasaligqvt LssrlsdGin
HEV eIqIpeFTI gnleEFIQts spkVtIDCaa fVcGdyaaCq qqLaeYgSfC DnInaiLiev neLldttqlQ vanslmngvt LstklkdGin
BCoV eIqIpeFTI gnmvEFIQts spkVtIDCaa fVcGdyaaCq 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-CoVgf..nf sqil..... .pdplkpt k...RSFIED LLFknVtLad aGfmkq.Yge ClGdinarDL ICaQkFNGLT VLPplltddm
HCoV-229E ynlsVi... ps..... .lptsgS rvagRSAIED ILFsKlvtSg lgtVdadYkK CTkGlsIaDL aCaQyYNGIm VLPgvadaer
TGEV etlDpIykw psigGswlwg lkdilpshns krkygSAIED LLFdkVvtSg lgtVdedYkr CTGGyDIaDL VCaQyYNGIm VLPgvanaek
FIPV enlDpIykw psigGswlwg lkdilpshns krkygSAIED LLFdkVvtSg lgtVdedYkr CTGGyDIaDL VCaQyYNGIm VLPgvanaek
HCoV-OC43 spvlGclgse cska.....S ...RSAIED LLFdkVklSd vGfVea.Ynn CTGGAeIrDL ICvQsYKGIk VLPpllsenq isgYtlaats
MHV gqiDdI..nf spllGclgsd cgevtmaaq g...RSAIED vLFDkVklSd vGfVea.Ynn CTGGQeVrDL LcVQsFNGIk VLPpvllsenq
HEV fnvDdI..nf ssvlGclgse cnra.....S t...RSAIED LLFdkVklSd vGfVea.Ynn CTGGAeIrDL ICvQsYNGIk VLPpllsenq
BCoV fnvDdI..nf spvlGclgsd cnkv.....S ...RSAIED LLFdkVklSd 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 iaaYTaalsv gtatagwtfG aGaalqiPfa MqmayRfngi gvtqnVlyeN QkQIAnqFNr AisqIqEsIt tts..... .TAL
HCoV-229E mamYTgslig gIal...ggl .tsavsiPFs LaiQaRLNv alqtDVLqen QkiLaasFNk AmtnIvDaft gvNdaitqts qalqtvatAL
TGEV mtmYTaslag gitl...gAl gGavaiPfa vaVQaRLNv alqtDVLnkn QqiLasAFNq AignItQsFg kvNdaihqts rglatvakAL
FIPV mtmYTaslag gitl...gAl gGavaiPfa vaVQaRLNv alqtDVLnkn QqiLanAFNq AignItQaFg kvNdaihqts gglatvakAL
HCoV-OC43 aslfpwptAa aG...vPFy LnVQyRINGl gvtmDVLsqN QkLIAnAFNn AlyaIqEgFd atN..... .SAL vKIQaVVNan
MHV isgYTagatw samf.pwsAa aG...vPFs LsVQyRINGl gvtmDVLseN QkMIAsAFNn AigaIqEgFa atN..... .SAL
HEV isgYTaata aslfpwptAa aG...vPFy LnVQyRINGl gvtmDVLsqN QkLIAsAFNn AldsIqEgFd atN..... .SAL
BCoV isgYtlaats aslfpwptAa 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 LsnFgAIsS vLnDilsRLD kvEAEvQIDR LIitGRLqsLq tyVtQqLira aeIraSanla atKmsECVlg
HCoV-229E nKIQdVVNqg gnsLnhLtsQ LrqnFqAIsS SIqIdyRDL tIQADQVDR LIitGRLaALN vFVshLtky teVraSrqla qQKNECVKS
TGEV akVQdVVNtq gQALshLtvQ LqNnFqAIsS SIsDIynRLD eLSADAhVDR LIitGRLTALN AFVSQtLtrq aeVraSrqla kDKVNECVKS
FIPV akVQdVVNtq gQALshLtvQ LqNnFqAIsS SIsDIynRLD eLSADAhVDR LIitGRLTALN AFVSQtLtrq aeVraSrqla kDKVNECVKS
HCoV-OC43 aEALNnLlqQ LsNrFgAIsA SLqEILsRLD aLEAQAIDR LIInGRLTALN AYVSQqLsds tlvKfSaaqA mEKVNECVKS
MHV aKmQfVVNan aEALNnLlqQ LsNrFgAIsA SLqEILsRLD aLEAQAIDR LIInGRLTALN AYVSQqLsds tlvKvSaaqA iEKVNECVKS
HEV vKIQaVVNan aEALNnLlqQ LsNrFgAIsA SLqEILsRLD aLEAQAIDR LIInGRLTALN AYVSQqLsds tlvKfSaaqA iEKVNECVKS
BCoV vKIQaVVNan aEALNnLlqQ LsNrFgAIsS SLqEILsRLD aLEAQAIDR LIInGRLTALN 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

Fig. 2. (continued)

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1251
SARS-CoV QSKRvdFCGk GyHLmSfpqa AphGvvFLHv tyvPsqernf ttapaIChe. GE...ayfpR e...gvFvfn gtsWfiTqrn fFsPqiiTtd
HCoV-229E QSKRygfFCGN GtHIfSIvna APEGLvFLHt vllPTaYkdv eawsGLCv.. .DgtnGyv1R qpnalalyke. gnyYriTsri mFePripTma
TGEV QSqRfgFCGN GtHLfSLana APnGMiFfHa vllPTaYeTv tawaGICald GDrtfGlvvK dvqltLFrnl ddkfy1Tprt mYqPrvaTss
FIPV QSqRfgFCGN GtHLfSLana APnGMiFfHt vllPTaYeTv tawsGICasd GDrtfGlvvK dvqltLFrnl ddkfy1Tprt mYqPrvaTss
HCoV-OC43 QSSRinFCGN GnHiiSLvqn APyGLyFIHf syvPTkYvTa rvspGLCia. GDr..GiapK s...gyFvvn nntWmyTgsg yYyPepiTgn
MHV QSSRinFCGN GnHiiSLvqn APyGLyFIHf syvPTsFtTa nvspGLCis. GDr..GlapK a...gyFvqd dgeWkftGsn yYyPepiTdk
HEV QSSRinFCGN GnHiiSLvqn APyGLyFIHf syvPTkYvTa kvspGLCia. GDi..GispK s...gyFinv nnsWmfTgsg yYyPepiTgn
BCoV QSSRinFCGN GnHiiSLvqn APyGLyFIHf syvPTkYvTa kvspGLCia. GDr..GiapK s...gyFvvn 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
SARS-CoV ntfvsgnCdv vigiinntvy dplqPeldsf k...EELdky FKnhts..PD vdlgdisgIn asvvnIqkEi d..... RLsev
HCoV-229E dfVqienCnV tFvnirsrseL qtivPeyiDv nktlQEL.s YK1pnytvPD 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 vTfLdLqEm n..... RLqea
MHV nsVvmssCaa nYtkApevfL ntsiPnlpDf k...EELdkw FKnqtsiaPD Lsl.dFek1N vTfLdLdEm n..... RIqda
HEV nvVvmstCav nYtkApdml ntstPnlpDf k...EELyqw FKnqsslapD Lsf.dY..iN vTfLdLqEm n..... RLqea
BCoV nvVvmstCav nYtkApdvmL nistPnlpDf k...EELdqw FKnqtsvaPD Lsl.dY..iN vTfLdLqEm n..... RLqea
Consensus --V----C-V -Y--A----L ----P---D- ----EEL--- FK-----PD L----Y---N -T-L-L--E-----RL---

1431
SARS-CoV aknLNeslId LqeLgkyEqY IKWPPWYVWLc fIaqlIaiym vtilLCCmTs C.csc1kgaac sCgsCCKfde ddsepvLkay klhvt~
HCoV-229E IdnINstlVd LkwLnrvEtY IKWPPWYVWLc IsvvlIifvvs MLllcCCsTG Ccgffscfas sirgCCestk lpyyd.Veki hiq~~~
TGEV IdnINnt1VN LewLnriEtY VKWPPWYVWLL IgLvVfCip LLLfCCsTG Ccgcgic1gs cChsiCsrrq fenypIekv hih~~~
FIPV IdnINnt1VN LewLnriEtY VKWPPWYVWLL IgLvVfCip LLLfCCsTG Ccgcgic1gs cChsiCsrrq fenypIekv hvh~~~
HCoV-OC43 IkvLNqsyIN LkdIgtYeyY VKWPPWYVWLL IcLagVamLv LLfFiCCcTG Cgtscfk...kCggCCddyt gyqelVI... ktshdd
MHV IkkLNesyIN LkdvgtyEmY VKWPPWYVWLL IgLagVavcv LLfFiCCcTG Cgscfk...kCgnCCdecg ghqdsiVihN issued
HEV IkvLNqsyIN LkdIgtYeyY VKWPPWYVWLL IcLagVamLv LLfFiCCcTG Cgtscfk...kCggCCddyt ghqelVI... ktshdd
BCoV IkvLNqsyIN LkdIgtYeyY VKWPPWYVWLL IgLagVamLv LLfFiCCcTG Cgtscfk...kCggCCddyt ghqelVI... ktshdd
Consensus I--LN---IN L--L--E--Y VKWPPWYVWLL 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'-azino-bis(3-ethylbenzthiazol-

line-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

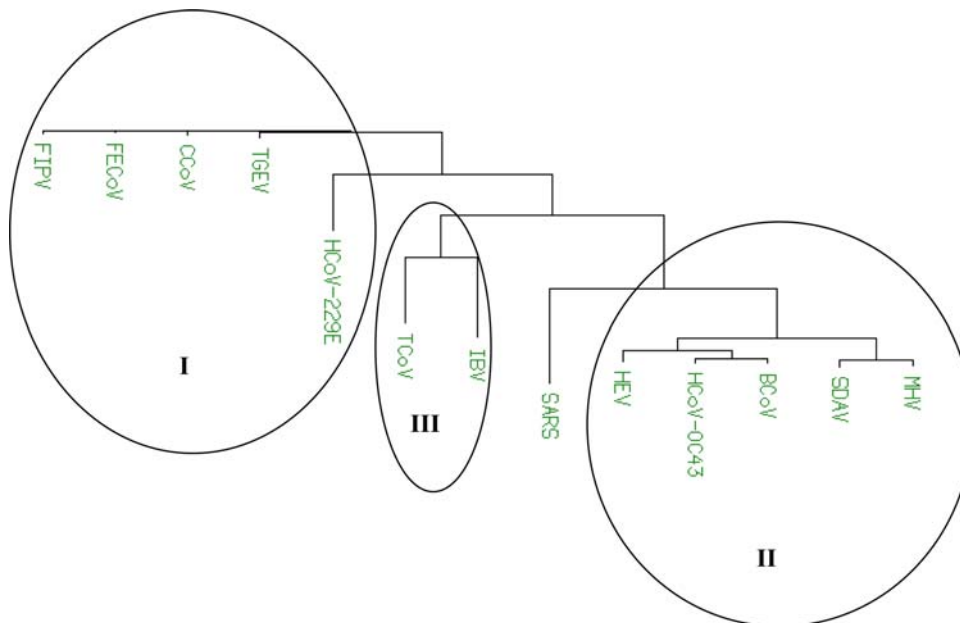


Fig. 3. Phylogenetic analysis of coronavirus S proteins. Amino acid sequences of SARS-CoV S proteins were compared with those of HCoV-229E, TGEV, FIPV, HCoV-OC43, MHV, HEV, BCov, avian infectious bronchitis virus (IBV) (GenBank Accession No. P11223), and turkey coronavirus (TCoV) (GenBank Accession No. 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.

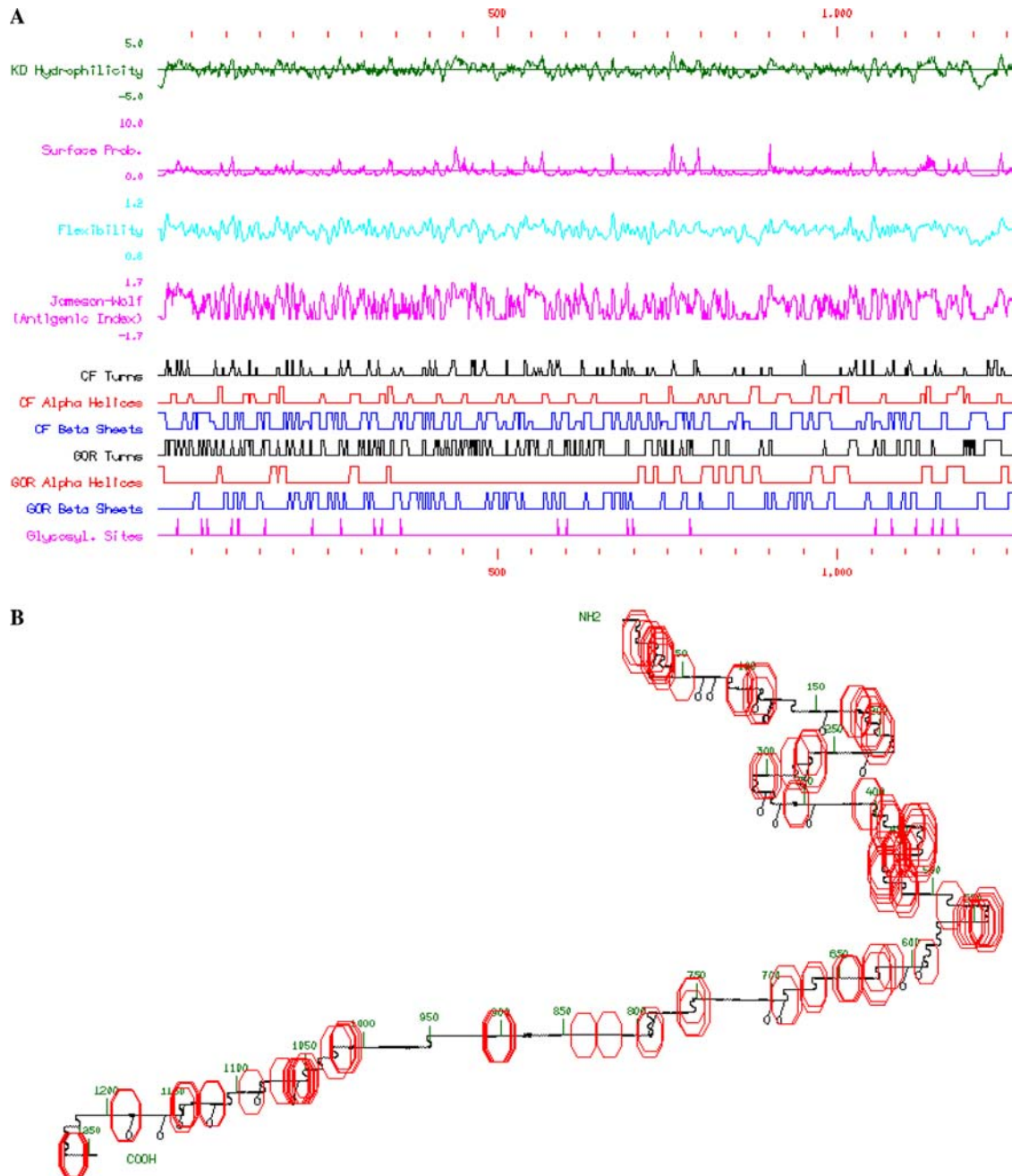


Fig. 4. Analysis of the primary sequence of SARS-CoV S protein. (A) The hydrophilicity, surface probability, and chain flexibility of SARS-CoV S protein were analyzed by PeptideStructure program of GCG. (B) Antigenic analysis of SARS-CoV S protein. The secondary structure and putative antigenic sites 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 dena-

tured 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

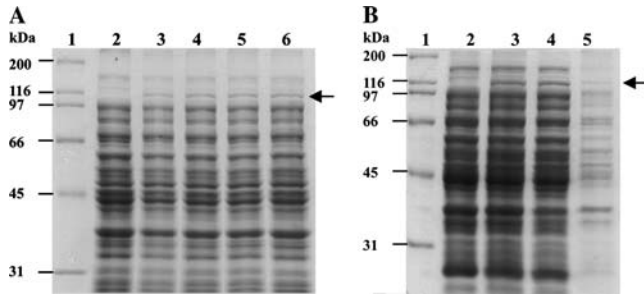


Fig. 5. Optimal condition and distribution of recombinant SARS-CoV S protein in *E. coli*. (A) Optimal condition for expressing recombinant S protein. The *E. coli* BL21(DE3)pLysS strain containing pET-spike was inoculated at 37 °C with shaking. After an overnight incubation, the bacteria were refreshed to 0.45 (lane 3), 0.5 (lane 4), 0.55 (lane 5) or 0.6 OD₆₀₀ (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.

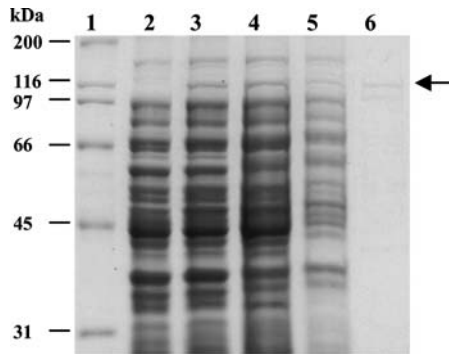


Fig. 6. SDS–PAGE analysis of recombinant SARS-CoV S protein. The total proteins (lane 3), soluble (lane 4), unbinding (lane 5), and purified fractions (lane 6) of induced *E. coli* were analyzed by 10% SDS–PAGE and stained by Coomassie brilliant blue. Lane 2 represents the preparation of uninduced *E. 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

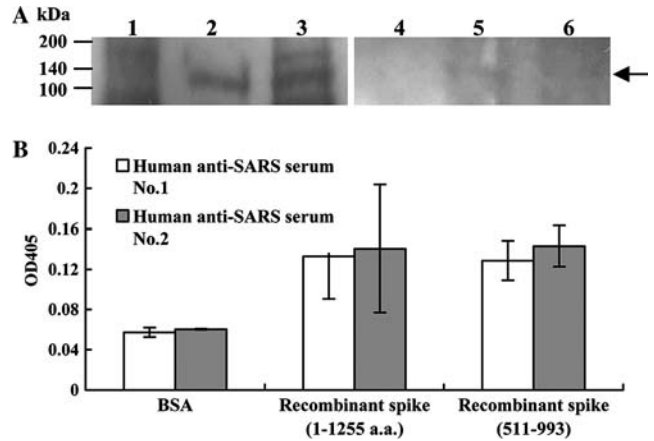


Fig. 7. Antigenic analysis of recombinant SARS-CoV S protein. (A) Western blot analysis. The preparations of uninduced *E. coli* (lanes 1 and 4), induced *E. coli* (lanes 3 and 6), and purified S protein (lanes 2 and 5) were analyzed by 10% SDS–PAGE, transferred to nitrocellulose membrane, detected by 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 \pm standard error of triplicate assays.

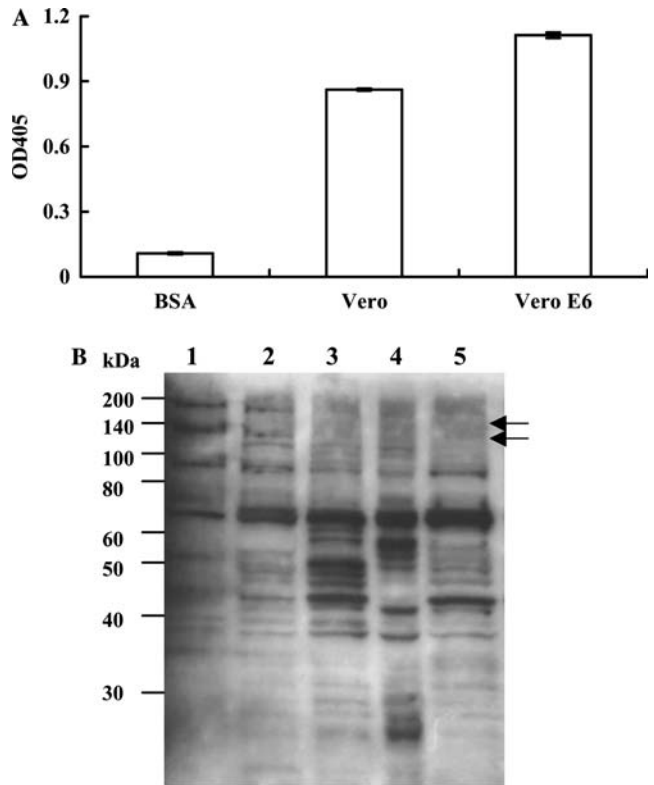


Fig. 8. Receptor-binding ability of recombinant S protein. (A) Biotinylated ELISA. The BSA, Vero cell extract, and Vero E6 cell extract were coated on ELISA plates and detected by biotin-labeled S protein. Values are means \pm standard error with triplicate assays. (B) Biotinylated Western blot. The 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 OD₄₀₅ 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 OD₄₀₅ 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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