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Difference in Receptor Usage between Severe Acute Respiratory Syndrome (SARS) Coronavirus and SARS-Like Coronavirus of Bat Origin▽

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Severe acute respiratory syndrome (SARS) is caused by the SARS-associated coronavirus (SARS-CoV), which uses angiotensin-converting enzyme 2 (ACE2) as its receptor for cell entry. A group of SARS-like CoVs (SL-CoVs) has been identified in horseshoe bats. SL-CoVs and SARS-CoVs share identical genome organizations and high sequence identities, with the main exception of the N terminus of the spike protein (S), known to be responsible for receptor binding in CoVs. In this study, we investigated the receptor usage of the SL-CoV S by combining a human immunodeficiency virus-based pseudovirus system with cell lines expressing the ACE2 molecules of human, civet, or horseshoe bat. In addition to full-length S of SL-CoV and SARS-CoV, a series of S chimeras was constructed by inserting different sequences of the SARS-CoV S into the SL-CoV S backbone. Several important observations were made from this study. First, the SL-CoV S was unable to use any of the three ACE2 molecules as its receptor. Second, the SARS-CoV S failed to enter cells expressing the bat ACE2. Third, the chimeric S covering the previously defined receptor-binding domain gained its ability to enter cells via human ACE2, albeit with different efficiencies for different constructs. Fourth, a minimal insert region (amino acids 310 to 518) was found to be sufficient to convert the SL-CoV S from non-ACE2 binding to human ACE2 binding, indicating that the SL-CoV S is largely compatible with SARS-CoV S protein both in structure and in function. The significance of these findings in relation to virus origin, virus recombination, and host switching is discussed.

The outbreaks of severe acute respiratory syndrome (SARS) in 2002–2003, which resulted in over 8,000 infections and close to 800 deaths, was caused by a novel coronavirus (CoV), now known as the SARS-associated CoV (SARS-CoV) (12, 25, 33, 36). The association of SARS-CoV with animals was first revealed by the isolation and identification of very closely related viruses in several Himalayan palm civets (*Paguma larvata*) and a raccoon dog (*Nyctereutes procyonoides*) at a live-animal market in Guangdong, China. A very high genome sequence identity (more than 99%) exists between the SARS-CoV-like virus from civets and SARS-CoV from humans, supporting the notion that SARS-CoV is of animal origin (18). However, subsequent studies showed that palm civets on farms and in the field were largely free from SARS-CoV infection (23, 40). These results suggested that palm civets played a role as an intermediate host rather than as a natural reservoir. Subsequent surveillance studies among different bat populations revealed the presence in several horseshoe bat species (genus *Rhinolophus*) of a diverse group of CoVs, which are very similar to SARS-CoV in genome organization and sequence. These viruses are designated SARS-like CoVs (SL-CoVs) or SARS-CoV-like viruses, (26, 29). Such discoveries raised the possibility that bats are the natural reservoirs of SARS-CoV (26, 29, 38) and triggered a surge in the search for CoVs in different bat species in different geographic locations (39, 43, 44a).

Phylogenetic analysis based on different protein sequences suggested that SL-CoVs found in bats and SARS-CoVs from humans and civets should be placed in a separate subgroup (group b) in CoV group 2 (G2b) to differentiate them from other group 2 CoVs in the genus *Coronavirus* (17, 26, 29, 43). G2b CoVs display major sequence differences in the N-terminal regions of their S proteins. The S proteins of CoVs play a key role in virus entry into host cells, including binding to host cell receptors and membrane fusion (4, 10, 24). Angiotensin-converting enzyme 2 (ACE2) has been identified as the functional receptor of SARS-CoV, and the molecular interaction between ACE2 and the SARS-CoV S protein has been well characterized (27, 28, 31, 42). A 193-residue fragment (amino acids [aa] 318 to 510) in the SARS-CoV S protein was demonstrated to be the minimal receptor-binding domain (RBD) which alone was able to efficiently bind to ACE2 (1, 42a, 45). Furthermore, it was shown that minor changes in amino acid residues of the receptor-binding motif (RBM) of SARS-CoV S

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protein could abolish the entry of SARS-CoV into cells expressing human ACE2 (huACE2) (7, 31). In the corresponding RBD region of the SL-CoV S proteins, there is significant sequence divergence from those of the SARS-CoV S proteins, including two deletions of 5 and 12 or 13 aa. From crystal-structural analysis of the S-ACE2 complex, it was predicted that the S protein of SL-CoV is unlikely to use huACE2 as an entry receptor (30), although this has never been experimentally proven due to the lack of live SL-CoV isolates. Whether it is possible to construct an ACE2-binding SL-CoV S protein by replacing the RBD with that from SARS-CoV S proteins is also unknown.

In this study, a human immunodeficiency virus (HIV)-based pseudovirus system was employed to address these issues. Our results indicated that the SL-CoV S protein is unable to use ACE2 proteins of different species for cell entry and that SARS-CoV S protein also failed to bind the ACE2 molecule of the horseshoe bat, *Rhinolophus pearsonii*. However, when the RBD of SL-CoV S was replaced with that from the SARS-CoV S, the hybrid S protein was able to use the huACE2 for cell entry, implying that the SL-CoV S proteins are structurally and functionally very similar to the SARS-CoV S. These results suggest that although the SL-CoVs discovered in bats so far are unlikely to infect humans using ACE2 as a receptor, it remains to be seen whether they are able to use other surface molecules of certain human cell types to gain entry. It is also conceivable that these viruses may become infectious to humans if they undergo N-terminal sequence variation, for example, through recombination with other CoVs, which in turn might lead to a productive interaction with ACE2 or other surface proteins on human cells.

MATERIALS AND METHODS

Cell lines and antibodies. The human cell lines 293T and HeLa were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco). Goat polyclonal antibody against the huACE2 ectodomain was purchased from R&D Systems. Monoclonal antibody (MAb) F26G8, which recognized a linear epitope (aa 615 to 620 of the SARS-CoV S protein) conserved in all known S proteins of SARS-CoVs and SL-CoVs (M. Yu, unpublished results), was kindly provided by Jody Berry (3). A MAb against p24 of HIV was generated by the HIV group of the Wuhan Institute of Virology (unpublished results). Rabbit polyclonal antibodies against ACE2 of the bat *R. pearsonii* (RpACE2) was generated using a recombinant RpACE2 protein expressed in *Escherichia coli* at our laboratory at the Wuhan Institute of Virology, following standard procedures. Alkaline phosphatase (AP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibodies were purchased from Santa Cruz Biotechnology, AP-conjugated goat anti-rabbit IgG from Chemicon (Australia), AP-conjugated protein-A/G mix from Pierce, and fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG from PTGLab (Chicago, IL).

Construction of expression plasmids. The construction of a codon-optimized spike (S) protein gene of SARS-CoV BJ01 (BJ01-S) in pcDNA3.1(+) was described previously (34, 46). The full-length S gene of a bat SL-CoV (Rp3) was cloned by PCR amplification from cDNA prepared using fecal samples from an *R. pearsonii* bat positive for SL-CoV (29). After codon optimization for the first 400 aa at the N terminus, the modified S gene was cloned into pcDNA3.1(+). For introduction of the RBM of SARS-CoV S into the SL-CoV S, the coding region from aa 424 to 494 of BJ01-S was used to replace the corresponding regions of Rp3-S, resulting in a chimeric S (CS) gene designated $CS_{424\text{-}494}$. Using the same strategy, a series of CS genes with BJ01-S sequences were constructed by stepwise replacement. To facilitate the construction of S chimeras, a point mutation (A to G) at nucleotide 1825 (the A residue of the ATG codon was designated nucleotide 1) was introduced to generate a unique EcoRI site in the S open reading frame. The chimera $CS_{424\text{-}494}$ was confirmed by full-length sequencing to ensure that no unexpected mutation was introduced during the PCR processes. For other chimeras constructed using $CS_{424\text{-}494}$ as a donor plasmid, only the newly inserted sequences between the BamHI (at the 5′ end) and EcoRI (at nucleotide 1825) sites were confirmed by direct sequencing. The amino acid numbering of BJ01-S was used for all chimeric constructs in this study.

Cloning of ACE2 genes and establishment of ACE2-expressing cell lines. The coding region for the ACE2 homolog in the horseshoe bat *R. pearsonii* was obtained from the intestine of a bat infected with SL-CoV by PCR using the following primers: 5′-CGGATCCGCCACCATGTCAGGCTCTTTCTGGC-3′ (upstream primer containing a BamHI site [underlined]) and 5′-CGTCGACCT AAAAGGAGGTCTGAACATCATCA-3′ (downstream primer with a SalI site [underlined]). Cell lines expressing huACE2 and palm civet (pcACE2) proteins were established in a previous study (37). The bat ACE2 coding region was cloned into a retroviral vector, pBabe-pure, and then transduced into HeLa cells to generate stable cell lines expressing the bat ACE2 by following the same procedures described previously (37). The stable expression of ACE2 in the transduced HeLa cells was confirmed by Western blotting, immunofluorescence, and ACE2 activity assays.

Construction and purification of pseudoviruses. HIV-based pseudoviruses were prepared as described previously (37). In brief, 12 μg each of pHIV-Luc (pNL4.3.Luc.R^-E^--Luc) and the S-expressing plasmids (or empty vector control) was cotransfected into 2×10^6 293T cells in 10-cm dishes. After 8 h, the medium was replaced with fresh medium. Supernatants were harvested at 48 h posttransfection, clarified from cell debris by centrifugation at 3,000 × *g*, and filtered through a 0.45-μm-pore-size filter (Millipore). Pseudoviruses in the cell supernatant (4 ml) were purified by ultracentrifugation through a 20% sucrose cushion (4 ml) at 55,000 × *g* for 60 min using a Ty90 rotor (Beckman). The pelleted pseudoviruses were dissolved in 100 μl of phosphate-buffered saline (PBS) and stored at −80°C in aliquots until they were used. To evaluate the incorporation of S proteins and HIV p24 into the pseudotyped viruses, 20 μl of purified viruses was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as detailed below.

Analysis of S protein expression and pseudovirus packaging by Western blotting. Lysates of 293T and HeLa cells or pelleted pseudovirions were separated by SDS-PAGE (using 8% gels), followed by transfer to a nitrocellulose membrane (Millipore). For Western blotting, incubation with different antibodies was done at room temperature for 1 h. For detection of S expression, the membrane was incubated with MAb F26G8 (1:1,000), and bound antibodies were detected using the AP-conjugated goat anti-mouse IgG, also diluted at 1:1,000. To detect HIV p24 in pseudoviruses, a MAb against HIV p24 (p24 MAb) was used as the first antibody at a dilution of 1:1,000, followed by the same AP-conjugated antibody described above. Expression of ACE2 in HeLa cells was detected using either the goat antibody against the huACE2 ectodomain (1:500) or the rabbit antibody against bat RpACE2 (1:100) as a primary antibody, followed by incubation with the AP-conjugated protein-A/G mix (1:1,000) or AP-conjugated goat anti-rabbit IgG (1:1,000).

Examination of pseudovirus virions by EM. Purified pseudoviruses were checked by electron microscopy (EM) using Formvar- and carbon-coated copper grids (200 mesh), negatively stained with 2% phosphotungstic acid (pH 7.0), and examined at 75 kV in a Hitachi H-7000FA transmission electron microscope.

Immunofluorescence confocal microscopy. ACE2-expressing and control HeLa cells were grown on coverslips in 24-well plates (Costar, Corning, NY). The following day, the cells were washed with PBS and fixed with 4% formaldehyde in PBS (pH 7.4) for 30 min at 4°C, followed by overnight incubation with goat anti-huACE2 antibody (at 1:150 dilution in PBS) at 4°C. The next day, incubation with FITC-conjugated donkey anti-goat IgG (at 1:100 dilution in PBS) was carried out at room temperature for 30 min. For nuclear staining, cells were incubated with Hoechst 33258 for 5 min at room temperature. Each incubation step was followed by washing with PBS five times. After the final wash, the slides were mounted with 50% glycerol and observed under a Leica confocal microscope (Leica, Germany).

ACE2 activity assay. The cell membrane fraction was prepared as described previously (11). Briefly, HeLa cells (with or without ACE2 expression) were collected by centrifugation at 10,000 × *g* for 5 min. The cell pellet was washed with ice-cold PBS and suspended in Tris-buffered saline (TBS) (100 mM Tris and 500 mM NaCl, pH 6.5). The cells were subjected to three cycles of freeze/thaw, followed by brief sonication on ice. The cell lysate was centrifuged at 15,000 × *g* for 30 min, and the supernatant was taken as the soluble protein fraction. The pellet, which contained the membrane fraction, was washed with ice-cold TBS and recentrifuged as described above. After resuspension in TBS, the total protein contents of both the membrane and soluble fractions were determined with a BCA Protein Assay Kit (Chenergy Biocolor, China) using bovine serum albumin as a standard. The activity of the ACE2 assay was determined as described by Douglas et al. (11) using the ACE2-specific quenched fluorescent

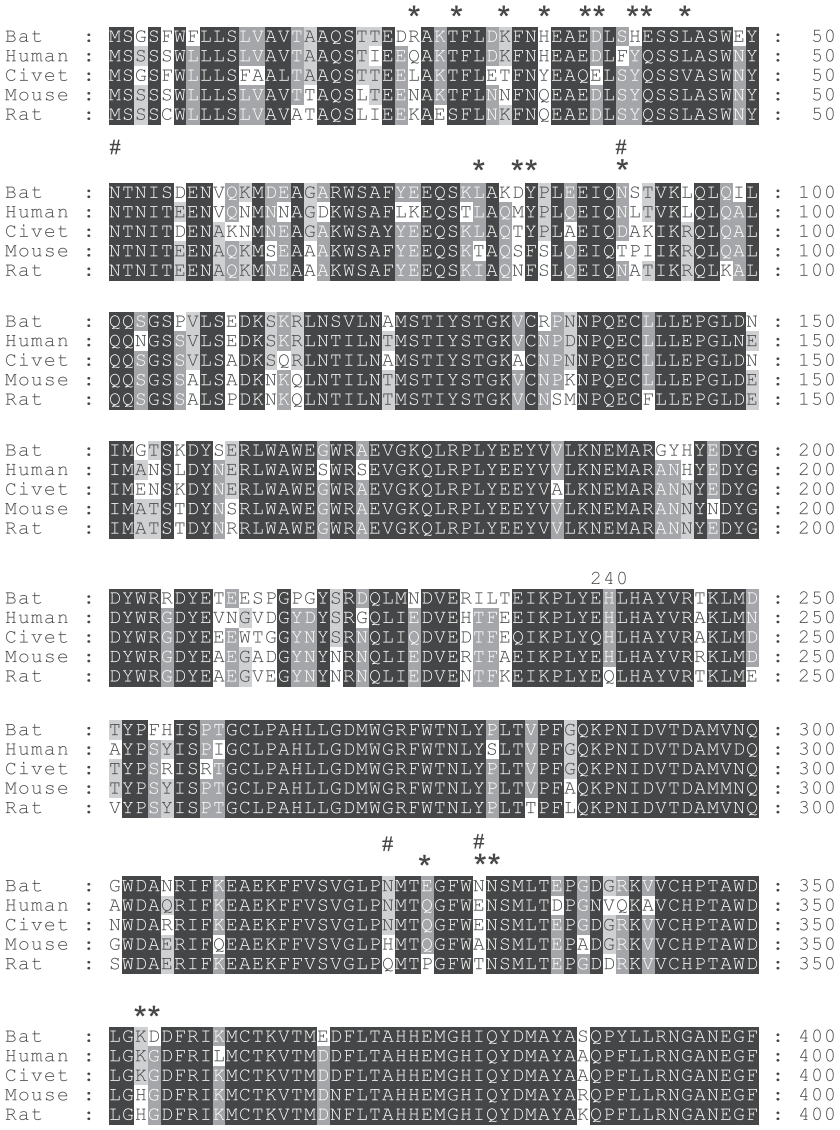

FIG. 1. Multiple amino acid sequence alignment of the N-terminal regions (aa 1 to 400) of different ACE2 proteins. The sequence alignment was conducted using the Clustal W program (39a). Potential N glycosylation sites are indicated by the # sign above the corresponding asparagine residues (N). Asterisks indicate the crucial amino acid residues that are involved in direct contact between the huACE2 and the SARS-CoV S protein. The accession numbers for the ACE2 proteins are as follows: human, NM 021804; palm civet, AY881174; RpACE2, EF569964; mouse, NM 027286; and rat, NM 001012006. Black shading, 100% identity; gray shading, 75% identity.

substrate QFS (7-methoxycoumarin-4-yl)-acetyl-Ala-Pro-Lys (2,4-dinitrophenyl), purchased from Auspep (Melbourne, Australia). Assays were performed in black 96-well plates with 50 μM QFS per reaction. Liberated fluorescence (in relative fluorescence units) after incubation at 37°C for 1 h was measured at 320 to 420 nm. To demonstrate specific inhibition, ACE2 activity was also determined in the presence of 0.1 mM EDTA.

Pseudovirus infection and infectivity assay. HeLa cells expressing huACE2, pcACE2, or bat RpACE2 were seeded in a 96-well plate at 1×10^4 cells/well 1 day before infection. For infection, 4 μl of purified pseudoviruses mixed with 96 μl of medium containing 8 μg/ml Polybrene was added to the cells. The mixture was removed and replaced with fresh medium 8 to 12 h postinfection (p.i.). The infection was monitored by measuring the luciferase activity, expressed from the reporter gene carried by the pseudovirus, using the luciferase assay system (Promega). Briefly, cells were lysed at 48 h p.i. by adding 20 μl of lysis buffer provided with the kit, and 10 μl of the resulting lysates was tested for luciferase activity by the addition of 50 μl of luciferase substrate in a Turner Designs TD-20/20 luminometer. Each infection experiment was conducted in triplicate, and all experiments were repeated three times.

GenBank accession number. The sequence of the *R. pearsonii* ACE2 coding region has been deposited in GenBank under the accession number EF569964.

RESULTS

Cloning of bat ACE2 and its functional expression in HeLa cells. The ACE2 gene homolog amplified from the horseshoe bat *R. pearsonii* (RpACE2) codes for an 805-aa protein that is identical in size to the ACE2 proteins of humans and civets. Sequence alignment indicated that the bat RpACE2 molecule is similar to the ACE2 proteins of other mammals and has an amino acid sequence identity of 81% to huACE2 and pcACE2 and 77% to mouse and rat ACE2 proteins. As shown in Fig. 1, the major sequence variation is located in the N-terminal region. For the 18 residues of huACE2 known to make direct

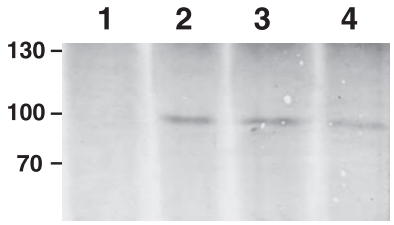

FIG. 2. Western blot analysis of bat ACE2 (RpACE2) expressed in HeLa cells using rabbit anti-RpACE2 antibodies. Lane 1, HeLa cell lysate used as a negative control; lanes 2 to 4, lysates from HeLa-huACE2, HeLa-pcACE2, and HeLa-RpACE2, respectively. The molecular masses (in kDa) of prestained protein markers (Fermentas, Canada) are given on the left.

contact with the SARS-CoV S protein (27), there are seven changes in RpACE2 compared to the cognate human protein: Q24R (Q in huACE2 and R in RpACE2 at aa 24), Y41H, Q42E, M82D, Q325E, E329N, and G354D (Fig. 1).

A stable HeLa cell line that continuously expressed the bat RpACE2 protein was established. Western blot analysis showed that RpACE2 was expressed efficiently in HeLa cells and was recognized by anti-RpACE2 antibodies (Fig. 2). Moreover, huACE2 and pcACE2 could also be recognized by anti-RpACE2 antibodies. The levels of expression for the three different ACE2 molecules were very similar. On SDS-PAGE, RpACE2 has mobility very similar to those of the other two ACE2 proteins, with an apparent mass of approximately 90 to 100 kDa, which is within the size range of the predicted mass of 93 kDa.

The localization of expressed ACE2 on the cell surface was demonstrated by immunofluorescence confocal microscopy using a commercial antibody raised against the ectodomain of huACE2 (Fig. 3). The surface location, as well as ACE2 functionality, was further assessed by an enzyme activity assay using an ACE2-specific peptide substrate, QFS. As shown in Fig. 4, membrane fractions prepared from three ACE2-expressing HeLa cell lines displayed substantially higher protease activities toward QFS than the control HeLa cells. Furthermore, the protease activity was largely abolished in the presence of 0.1 mM EDTA, a known inhibitor of ACE2 proteases (11). These data indicated that all three ACE2 molecules were expressed as a functional protease associated with cellular membranes.

Pseudovirus packaging. The expression of functional S proteins and their correct incorporation into pseudoviruses were monitored by three approaches, i.e., Western blotting of cell lysates with an S-specific MAb, Western blotting of pelleted virions using both S-specific and p24-specific MAbs, and direct examination of virions by EM. The results are summarized in Fig. 5. Both the BJ01-S and Rp3-S genes were efficiently expressed in transfected 293T cells, and the expressed S proteins were incorporated into the respective pseudoviruses as expected (Fig. 5A). When examined by EM, the pseudoviruses containing the Rp3-S or BJ01-S protein displayed a morphology characteristic of CoVs (Fig. 5B). On the other hand, contrasting results were obtained for two CS proteins. For CS_{14-608}, the expressed S protein was incorporated into the pseudovirus, as observed for the other two nonchimeric constructs. However, for $CS_{424-494}$, although the expression of the S protein

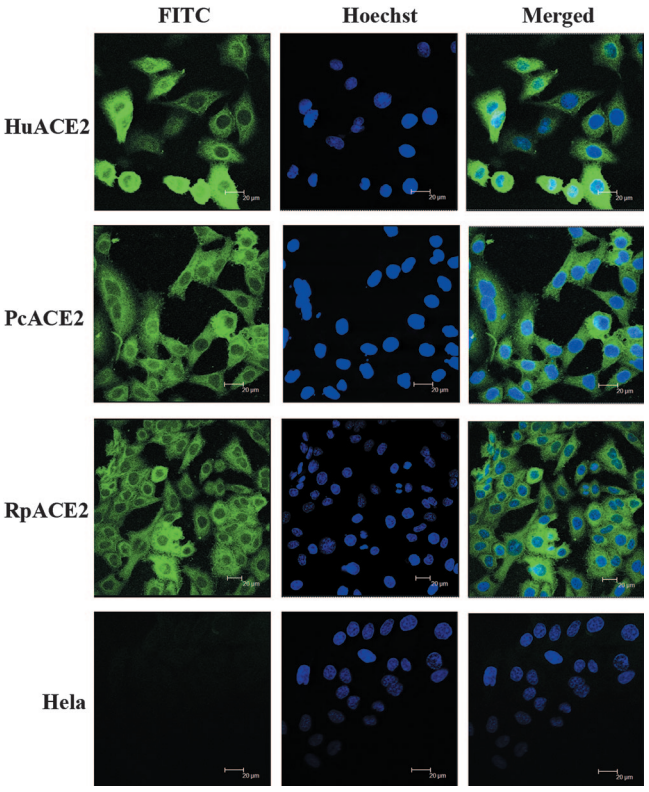

FIG. 3. Detection of ACE2 expression by immunofluorescence confocal microscopy. Cells were incubated with goat anti-huACE2 antibody, followed by probing with FITC-conjugated donkey anti-goat IgG. The top three rows (from the top down) show HeLa cells expressing huACE2, pcACE2, and RpACE2. The bottom row shows HeLa cells as a negative control. The columns (from left to right) show staining of expressed ACE2 (green fluorescence of FITC), staining of cell nuclei (blue fluorescence of Hoechst 33258), and the merged double-stained image.

was normal, the pseudovirus seemed unable to assemble the CS protein at a level detectable by Western blot analysis (Fig. 5A) and EM (Fig. 5B), whereas p24 expression and assembly appeared to be normal (Fig. 5A).

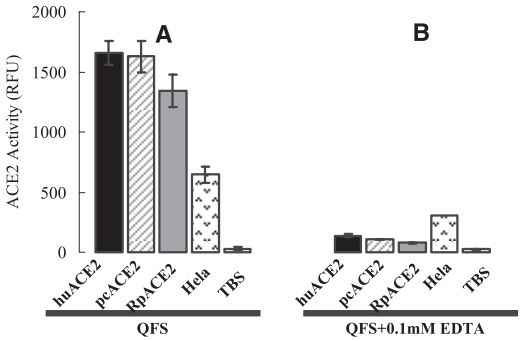

FIG. 4. Determination of ACE2 activity. The protease activities from different ACE2-expressing cell membrane fractions were determined using the ACE2-specific substrate QFS (see Materials and Methods). The liberated fluorescence (in relative fluorescence units [RFU]) determined at 320 to 420 nm in the absence (A) and presence (B) of EDTA is shown. HeLa cells with no exogenous ACE2 gene and PBS buffer were used as negative controls. The error bars indicate standard deviations.

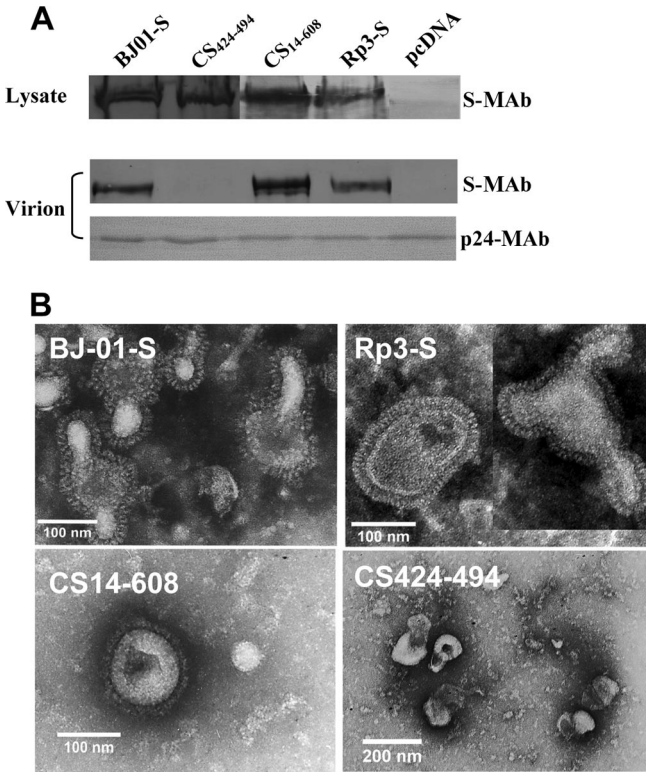

FIG. 5. Analysis of S protein expression and incorporation into pseudovirus. (A) Western blot analysis. S proteins expressed in transfected 293T cells were probed with MAb F26G8 or S-MAb (top); the middle and bottom panels are Western blots of pelleted pseudoviruses using MAbs F26G8 and p24, respectively. (B) EM examination of pseudovirus morphology. The name of the S protein construct in each pseudovirus is shown at the top left corner of the electron micrograph.

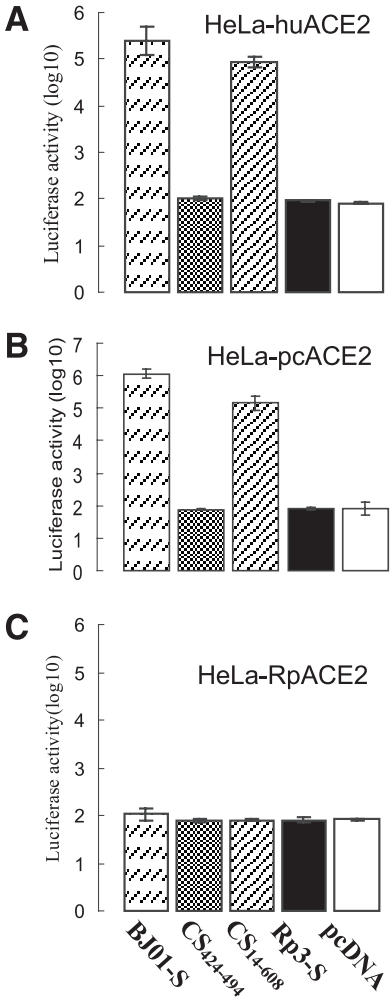

FIG. 6. Measurement of pseudovirus infectivity by determining the reporter luciferase activity. Cell lysates were prepared 48 h p.i. from HeLa-huACE2 (A), HeLa-pcACE2 (B), and HeLa-RpACE2 (C), and luciferase activity was determined as described in Materials and Methods. The error bars indicate standard deviations.

Use of huACE2, pcACE2, and bat RpACE2 for cell entry by different pseudoviruses. When HIV/Rp3-S pseudovirus was used to infect HeLa cells expressing huACE2, pcACE2, or RpACE2, only a low level of luciferase activity (approximately 100 relative light units, the same as the background level generated by the vector control) was obtained (Fig. 6). In contrast, when the BJ01-S-typed pseudovirus was used, high levels of luciferase activity (more than 10^5 relative light units) were detected in the cell lysates of HeLa-huACE2 and HeLa-pcACE2 (Fig. 6A and B), but not in HeLa-RpACE2 (Fig. 6C). Interestingly, the pseudovirus packaged with a CS protein, HIV/$CS_{14\text{-}608}$, displayed a level of luciferase activity similar to that of HIV/BJ01-S (Fig. 6A). As expected from the above-mentioned Western blot and EM analyses, HIV/$CS_{424\text{-}494}$, in which the RBM of BJ01-S was transferred into the Rp3-S backbone, failed to produce luciferase activity above the background level in any of the three ACE2-expressing HeLa cell lines.

Mapping of the minimal BJ01-S region required to convert the non-ACE2-binding Rp3-S to a chimeric ACE2-binding S protein. Based on the results mentioned above, it is evident that the Rp3-S protein is capable of mediating cell entry as long as the ACE2-binding component is incorporated into the N-terminal region of its molecule. To define the minimal region required for this conversion from non-huACE2 binding to huACE2 binding, a series of CS proteins was constructed, and the exact amino acid locations are summarized in Fig. 7A. S protein expression in 293T cells and its incorporation into pseudovirus for each construct were analyzed by Western blotting using S-specific and p24-specific antibodies as described above (Fig. 7B). Although the levels of S protein expression were similar for all constructs, significant differences in incorporation were observed. The S protein was undetectable in pseudoviruses derived from $CS_{371\text{-}608}$ and was only weakly detected for the chimeras $CS_{310\text{-}608}$, $CS_{45\text{-}608}$, and $CS_{310\text{-}518}$. The infectivities of all the CS constructs in HeLa-huACE2 were examined, and several observations were made (Fig. 7C). With the exception of the chimeras $CS_{417\text{-}608}$ and $CS_{371\text{-}608}$, which produced a background level of luciferase activity, all of the chimeras exhibited significant levels of luciferase activity, suggesting that pseudoviruses containing these CS proteins were able to use huACE2 for cell entry. From these results, it was deduced that the region from aa 310 to 518 of BJ01-S was necessary and sufficient to convert Rp3-S into a huACE2-

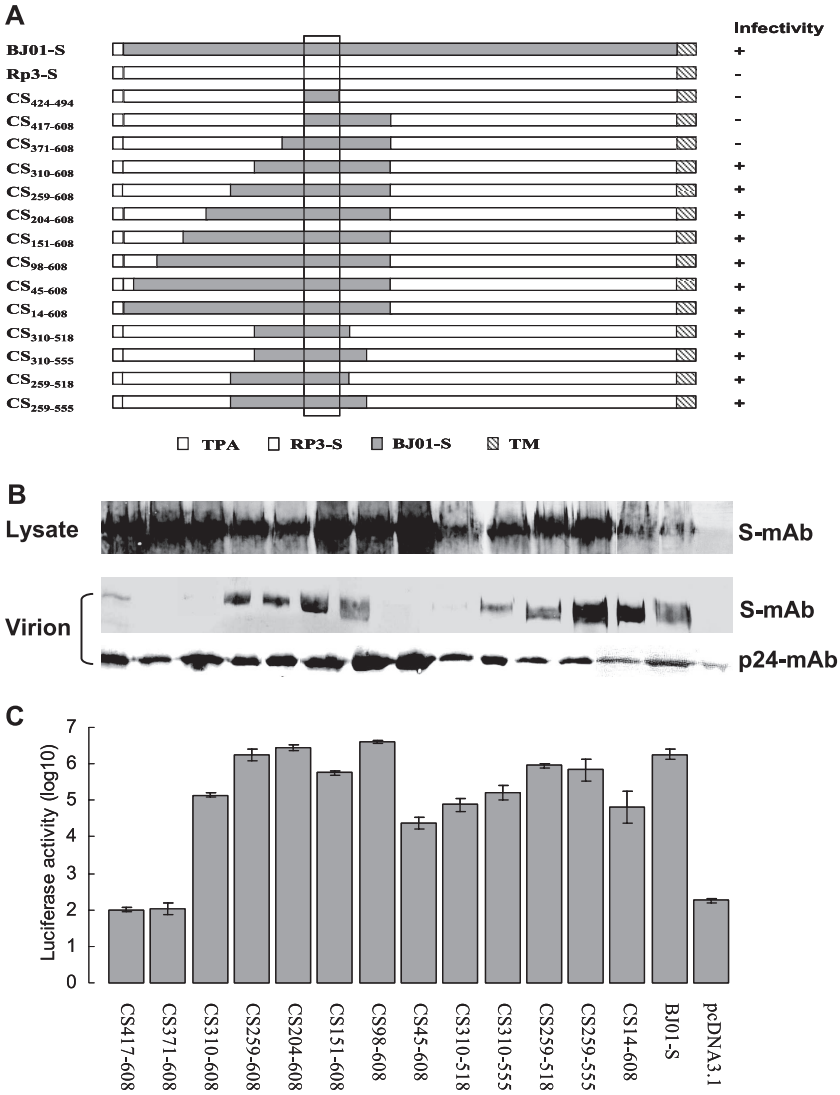

FIG. 7. Construction and functional analysis of pseudoviruses derived from different CS protein constructs. (A) Schematic presentation of constructs of human SARS-CoV S protein (BJ01-S), bat SL-CoV S (Rp3-S), and different CS proteins. The numbers in the subscripts indicate the amino acid locations of the BJ01-S sequences used to replace the corresponding region of Rp3-S. The open box indicates the location of the RBM. TPA, signal peptide from tissue plasminogen activator; TM, transmembrane domain derived from the fusion protein of Sendai virus. (B) Western blot analysis. S proteins in transfected 293T cells (top) or purified pseudoviruses (middle) were probed with MAb F26G8 (S-MAb); at the bottom is a blot of different pseudoviruses probed with p24 MAb as a control to determine the relative quantity of HIV pseudovirus for each construct. (C) Measurement of pseudovirus infectivity by determining luciferase activity. Cell lysates were prepared 48 h p.i. from HeLa-huACE2 infected with pseudoviruses containing different S proteins as indicated below the bar graph. The error bars indicate standard deviations.

binding molecule. For $CS_{310-608}$, CS_{45-608}, and $CS_{310-518}$, the levels of luciferase activity detected were much higher than that predicted from the level of S protein incorporated into the virion, suggesting that the infection assay is more sensitive than Western blotting. It is also important to note that although a low level of S protein was present in the virion of $CS_{417-608}$, it did not lead to a productive infection.

DISCUSSION

The CoV spike glycoproteins are responsible for cellular-receptor recognition, cell tropism, and host specificity (7, 9, 19, 25a). Our previous results showed that the SL-CoVs identified in bats are similar to SARS-CoV in genome sequence and organization. The key difference between these two groups of closely related viruses lies in their S protein sequences, specifically, the RBM, in which there are two deletions in the bat SL-CoV S sequences (29). From previous published studies indicating the importance of the structural fit between the receptor ACE2 and the S protein of SARS-CoV variants and the sensitivity of S proteins to point mutations in the RBM region, it was speculated that SL-CoV S is unlikely to use ACE2 as a receptor for cell entry unless the bat ACE2 homolog is significantly different from those of other mammals.

To address these unanswered questions, we cloned and expressed the bat *R. pearsonii* ACE2 gene and examined the abilities of ACE2 proteins from human, palm civet, and *R.*

pearsonii to support infection by HIV-based pseudoviruses containing different S protein constructs. Our results indicated that the bat SL-CoV (Rp3) S protein is unable to use ACE2 for cell entry regardless of the origin of the ACE2 molecule. We also demonstrated that the human SARS-CoV S cannot use bat RpACE2 as a functional receptor. On the other hand, we demonstrated that after replacement of a small segment (aa 310 to 518) of Rp3-S by the cognate sequence of BJ01-S, the CS protein mimics the function of BJ01-S in regard to receptor usage in the HIV pseudovirus assay system. Although we currently have no way of confirming that the Rp3-S protein is functional in binding its cognate receptor due to the lack of a horseshoe bat cell line, our chimeric-construct analysis suggests that the Rp3-S gene was intact and would be functional if an appropriate receptor was identified. It is also worth noting that, although we have no experimental data to demonstrate direct binding of different S proteins to huACE2 on the HeLa cell surface, we did confirm that either anti-huACE2 or anti-SARS-CoV polyclonal antibodies were able to neutralize infection by different pseudoviruses (data not shown).

The significance of these findings is as follows. First, the failure of SARS-CoV S protein to use bat RpACE2 as a receptor suggests that despite the presence of a diverse group of SL-CoVs in horseshoe bats, they are unlikely to be the natural reservoir of the immediate progenitor virus for SARS-CoV. It is therefore important to continue the search for the reservoir of SARS-CoV in other bat and wildlife species. It is also important to conduct such searches in different geographical locations because live-animal markets in southern China source their animals from all over China and from foreign countries (26, 29, 39) and it is possible that the natural host of SARS-CoV was not indigenous to the site of the first SARS outbreak. Second, as predicted, sequence variation in the N-terminal region of the SL-CoV S protein rendered it incapable of using ACE2 as a receptor for cell entry. However, the ACE2-binding activity of SL-CoVs was easily acquired by the replacement of a relatively small sequence segment of the S protein from the SARS-CoV S sequence, highlighting the potential dangers posed by this diverse group of viruses in bats. It is now well documented that bat species, including horseshoe bats, can be infected by different CoVs. Coinfection by different CoVs in an individual bat has also been observed (26, 29, 39). Knowing the capability of different CoVs to recombine both in the laboratory (2, 14, 15, 32) and in nature (22, 41, 44), the possibility that SL-CoVs may gain the ability to infect human cells by acquiring S sequences competent for binding to ACE2 or other surface proteins of human cells can be readily envisaged. This could occur if the same bat cells carry receptors for both types of viruses. In this context, it could be concluded that the particular horseshoe bat species investigated in the current study is unlikely to be the putative mixing host. However, one cannot rule out the possibility that a different horseshoe bat or a different bat species might have a functional ACE2, thus giving it the ability to act as a mixing host.

Our results indicated that the overall structure of the SL-CoV S protein is very similar to that of the SARS-CoV S protein. It remains to be seen whether a recombinant SL-CoV containing a CS protein (e.g., CS_{14-608}) will be capable of infecting experimental animals and causing disease. Such studies will be important to elucidate the molecular mechanism of pathogenesis for SARS-CoV and related viruses. The outcome of such research will also be invaluable in formulating control strategies for potential future outbreaks caused by viruses that are similar to, but different from, the SARS-CoVs responsible for the 2002–2003 outbreaks.

The RBM of SARS-CoV S protein has been localized between aa 424 and 494 by crystal structure reconstruction and functional studies (27). When this region alone was used to replace the cognate sequence in the SL-CoV S protein, the chimeric protein ($CS_{427-494}$) was unable to be incorporated into the pseudovirus at a level detectable by Western blotting. Similar results were obtained for a few other CS constructs. It is interesting that these CS constructs are similar to others in that the chimerization mutagenesis was located exclusively in the ectodomain. The failure of incorporation was thus most likely due to conformational changes that prevented the proper folding and cell surface presentation of the chimeric proteins. It is unclear at this stage whether these constructs can be functionally incorporated into a different pseudovirus system, such as the vesicular stomatitis virus system (16). At the same time, we also had CS constructs that showed very low levels of incorporation into virus but a significant level of luciferase activity or infection (e.g., CS_{45-608}). This suggests that there may not be a direct correlation between the efficiency of incorporation and cell entry, which argues for further investigation of these constructs using different systems.

The RpACE2 protein is composed of 805 aa, the same number as huACE2, and the level of sequence conservation among these two ACE2 proteins is significant, with 81% sequence identity and 90% similarity. Several published studies have identified a number of residues in huACE2 that are important for the S-ACE2 interaction (20, 27, 31). From crystal-structural analysis of an ACE2-S complex (20, 27, 31), 18 amino acid residues have been identified as being in direct contact with the SARS-CoV S protein. Among them, only seven are different in RpACE2 (Fig. 1). Residues Tyr-41 and Lys-353 have been shown to interact with amino acid residues of SARS-CoV S proteins that are most sensitive in S-ACE2 interaction (20, 27, 31). Since Lys-353 is conserved in RpACE2, it would be interesting to see whether the Tyr-to-His change (T41H) in RpACE2 was mainly responsible for the failure to act as a SARS-CoV receptor. It should be emphasized that, in addition to amino acid sequence variation, there are also differences in the numbers and locations of potential N glycosylation sites between huACE2 and RpACE2 (Fig. 1). It remains to be seen whether different glycosylation patterns also play a role in the utilization of ACE2 proteins of different species by SARS-CoVs.

Bats have been identified as natural reservoirs for many emerging zoonotic viruses, such as Hendra virus, Nipah virus, and lyssaviruses (6). Although an intermediate host is necessary to amplify most bat viruses and deliver them to human populations, it has been demonstrated that the Nipah virus in Bangladesh is capable of direct bat-to-human transmission (reviewed in reference 13). Since the discovery of SL-CoVs in bats, a large number of CoVs have been discovered in different bat species (26, 29, 35, 39, 43, 44a). It is now clear that bats are reservoirs of a diverse group of CoVs. Considering the documented observations of coinfection of the same bat species by

different CoVs, the same CoVs infecting different bat species (26, 29, 39), the high density of bat habitats, and the propensity for genetic recombination among different CoVs, it is not unreasonable to conclude that bats are a natural mixing vessel for the creation of novel CoVs and that it is only a matter of time before some of them cross species barriers into terrestrial mammal and human populations. The findings presented in this study serve as the first example of host switching achievable for G2b CoVs under laboratory conditions by the exchange of a relatively small sequence segment among these previously unknown CoVs.

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