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# The S proteins of human coronavirus NL63 and severe acute respiratory syndrome coronavirus bind overlapping regions of ACE2

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## Abstract

The cellular receptor for human coronavirus NL63 (HCoV-NL63), a group I coronavirus, is angiotensin-converting enzyme2 (ACE2). ACE2 is also the receptor for the SARS coronavirus (SARS-CoV), a group II coronavirus. Here we describe the ability of HCoV-NL63 to utilize a number of ACE2 variants previously characterized as SARS-CoV receptors. Several ACE2 variants that reduced SARS-CoV S-protein association similarly reduced that of HCoV-NL63, whereas alteration of a number of solvent-exposed ACE2 residues did not interfere with binding by either S protein. One notable exception is ACE2 residue 354, at the boundary of the SARS-CoV binding site, whose alteration markedly inhibited utilization by the HCoV-NL63 but not SARS-CoV S proteins. In addition, the SARS-CoV S-protein receptor-binding domain inhibited entry mediated by the HCoV-NL63 S protein. These studies indicate that HCoV-NL63, like SARS-CoV, associates region of human ACE2 that includes a key loop formed by  $\beta$ -strands 4 and 5.

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**Keywords:** Human coronavirus NL63; SARS coronavirus; Angiotensin-converting enzyme 2; Viral entry

## Introduction

Coronaviruses infect a wide range of hosts and cause enteric, respiratory, and neurotropic disorders in humans and animals (Bastien et al., 2005; Holmes et al., 1993; McIntosh, 2005). To date, five human coronaviruses from two phylogenetic groups have been characterized. Human coronaviruses HCoV-229E (group I), HCoV-OC43, and CoV-HKU1 (both group II) typically cause mild disease and are estimated to be responsible for 15–30% of common colds and can contribute to more serious lower respiratory tract infections (Woo et al., 2005). In contrast, the severe acute respiratory syndrome coronavirus (SARS-CoV) which emerged in the winter of 2002–2003 was

fatal in approximately 10% of infections (Bastien et al., 2005; Lee et al., 2003; Peiris et al., 2003; Zhong et al., 2003). Human coronavirus NL63 (HCoV-NL63, also described as HCoV-NL, HCoV-NH) was described in 2004 (Esper et al., 2005; Fouchier et al., 2004; van der Hoek et al., 2004). HCoV-NL63 infection appears to be common in childhood, and most adult sera contain antibodies that neutralize the virus (Hofmann et al., 2005; van der Hoek et al., 2004). In some cases, the virus can cause severe lower respiratory tract infections requiring hospitalization, especially of young children, the elderly, and immunocompromised adults (Arden et al., 2005; Bastien et al., 2005; Chiu et al., 2005; Ebihara et al., 2005; Gerna et al., 2006; Kaiser et al., 2005; Vabret et al., 2005). HCoV-NL63 has been also reported to be associated with croup (van der Hoek et al., 2005). Among coronaviruses, HCoV-NL63 is most similar to HCoV-229E, another group I coronavirus (Pyrce et al., 2004). However these viruses utilize distinct cellular receptors: HCoV-229E utilizes CD13, whereas HCoV-NL63 utilizes angiotensin-converting

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enzyme 2 (ACE2), the receptor for SARS-CoV, a group II coronavirus (Hofmann et al., 2005; Li et al., 2003; Yeager et al., 1992). The receptors for the other human group II coronaviruses HCoV-HKU1 and HCoV-OC43 have not been identified.

Spike (S) proteins of coronaviruses are large transmembrane glycoproteins that mediate receptor association, membrane fusion, and viral entry (Bosch et al., 2003; Colman and Lawrence, 2003; Gallagher and Buchmeier, 2001). The S proteins of some coronaviruses are cleaved into two subunits by cellular proteases (Jackwood et al., 2001; Sturman and Holmes, 1984; Sturman et al., 1985). HCoV-NL63, HCoV-229E, and SARS-CoV S proteins are not cleaved in producer cells, but two functionally distinct domains, corresponding to the subunits of cleaved S proteins (S1 and S2), have been described (Huang et al., 2006; Li et al., 2003; Xiao et al., 2003). The S1 domain mediates association with a cell-surface receptor, whereas the S2 domain contains the hydrophobic fusion peptide and coiled-coil regions that orchestrate the process of membrane fusion following receptor association (Bosch et al., 2003; Li et al., 2003; Xiao and Dimitrov, 2004).

Discrete, independently folded receptor-binding domains (RBDs) have been identified in the S1 domains of a number of coronaviruses (Bonavia et al., 2003; Breslin et al., 2003; Kubo et al., 1994; Wong et al., 2004). The first 330 amino acids of the spike protein of mouse hepatitis virus (MHV) efficiently bind the MHV receptor, CEACAM1 (Dveksler et al., 1991, 1993; Kubo et al., 1994); the RBD of the HCoV-229E S protein is located between residues 417 and 547 (Bonavia et al., 2003; Breslin et al., 2003); and a 193 amino-acid fragment of the SARS-CoV S1 domain, residues 318–510, binds human ACE2 with nanomolar affinity (Wong et al., 2004). The structure of the SARS-CoV RBD complexed to human ACE2 reveals an extended tyrosine-enriched loop of the RBD, residues 424–494, that are in direct contact with ACE2 (Li et al., 2005a). This region has been described as the receptor-binding motif (RBM) (Li et al., 2005a).

Here we show that alteration of residues in a region of the HCoV-NL63 S protein similar to the RBM region of SARS-CoV interfered with, and in one case, enhanced, association with ACE2. We also show that the S proteins of SARS-CoV and HCoV-NL63 bind overlapping regions of ACE2 that include a critical loop between its fourth and fifth  $\beta$ -strands. Our studies underscore some commonalities between the entry proteins of these two divergent coronaviruses and imply that some entry inhibitors of SARS-CoV may also be effective against HCoV-NL63.

## Results

### *HCoV-NL63 S1 truncation variants bind ACE2*

The S protein of HCoV-NL63 is composed of 1356 amino acids, including a 179-residue N-terminal fragment not present in other coronaviruses (Fouchier et al., 2004; van der Hoek et al., 2004). This fragment has been suggested to contribute to ACE2 recognition (Hofmann et al., 2005). To test this possibility, four truncation variants of the HCoV-NL63 spike

protein, encoding residues 16–200, 16–334, 16–749 and 198–749, were generated. These S1 variants were expressed as fusion proteins containing the Fc region of human IgG1 (S1-Ig), as previously described (Li et al., 2005c; Wong et al., 2004). Plasmids encoding these fusion proteins were transfected into HEK293T cells, which were subsequently incubated in 293SFM II medium for 48 h. Proteins were then purified with protein A–Sepharose 4B beads (Fig. 1A), and 1  $\mu$ g of each protein was incubated with HEK293T cells transfected with a plasmid expressing human ACE2. Transfected cells were then analyzed by flow cytometry. As shown in Fig. 1B, S1-Ig variants encoding residues 16–749 and 198–749 efficiently bound ACE2-expressing cells, whereas variants encoding 16–200 and 16–334 did not. Thus, important determinants of ACE2 association are located between residue 198 and 749, and the unique N-terminal region of HCoV-NL63 S protein does not contribute to receptor binding. These data are consistent with those previously reported by Hofmann et al. (2006). Fig. 1B also underscores the substantially higher efficiency with which SARS-CoV S protein binds ACE2, compared with that of HCoV-NL63.

To further define the receptor-binding region of the spike protein, additional truncation variants of S1-Ig were similarly made and characterized (Fig. 1C). Variants that included residues 198–701, 301–749, and 301–643 efficiently bound ACE2-expressing cells, whereas twelve additional smaller variants could not detectably bind these cells. The 301–643 variant consistently bound cells less efficiently than the 301–749 S1-Ig variant. Therefore residues between amino acids 301 and 643 make substantial contributions to ACE2 recognition, and those between amino acids 643 and 701 may also contribute, directly or indirectly, to receptor binding. Our subsequent assays utilized the 301–749 S1-Ig variant, described below as S1-Ig(301–749).

### *Alteration of asparagine 578 to tyrosine increases the affinity of HCoV-NL63 S1-Ig(301–749) for ACE2*

The structure of the SARS-CoV RBD complexed with human ACE2 has been determined (Li et al., 2005a). The RBD contains two subdomains, a five-stranded anti-parallel  $\beta$ -sheet core, and an extended loop including residues 424–494, described as the receptor-binding motif (RBM). Fourteen residues of the RBM, six of which are tyrosines, directly contact ACE2. A region of HCoV-NL63 S1, between residues 577 and 597, aligns with the SARS-CoV RBM (Fig. 2A). To determine the contribution of this region to ACE2 association, we converted several residues of S1-Ig(301–749) to their putative SARS-CoV equivalents. In most cases, these changes reduced ACE2 association. However, alteration of S1-Ig(301–749) asparagine 578 to tyrosine (N578Y) resulted in substantially more efficient binding to ACE2 (Fig. 2B). The consistent ability of changes in this region to alter the efficiency of ACE2 association suggests that this region of the HCoV-NL63 S protein contributes to ACE2 binding. Fig. 2C highlights SARS-CoV tyrosine 475, proposed here to be analogous to the N578Y alteration of HCoV-NL63 S1-Ig(301–749).

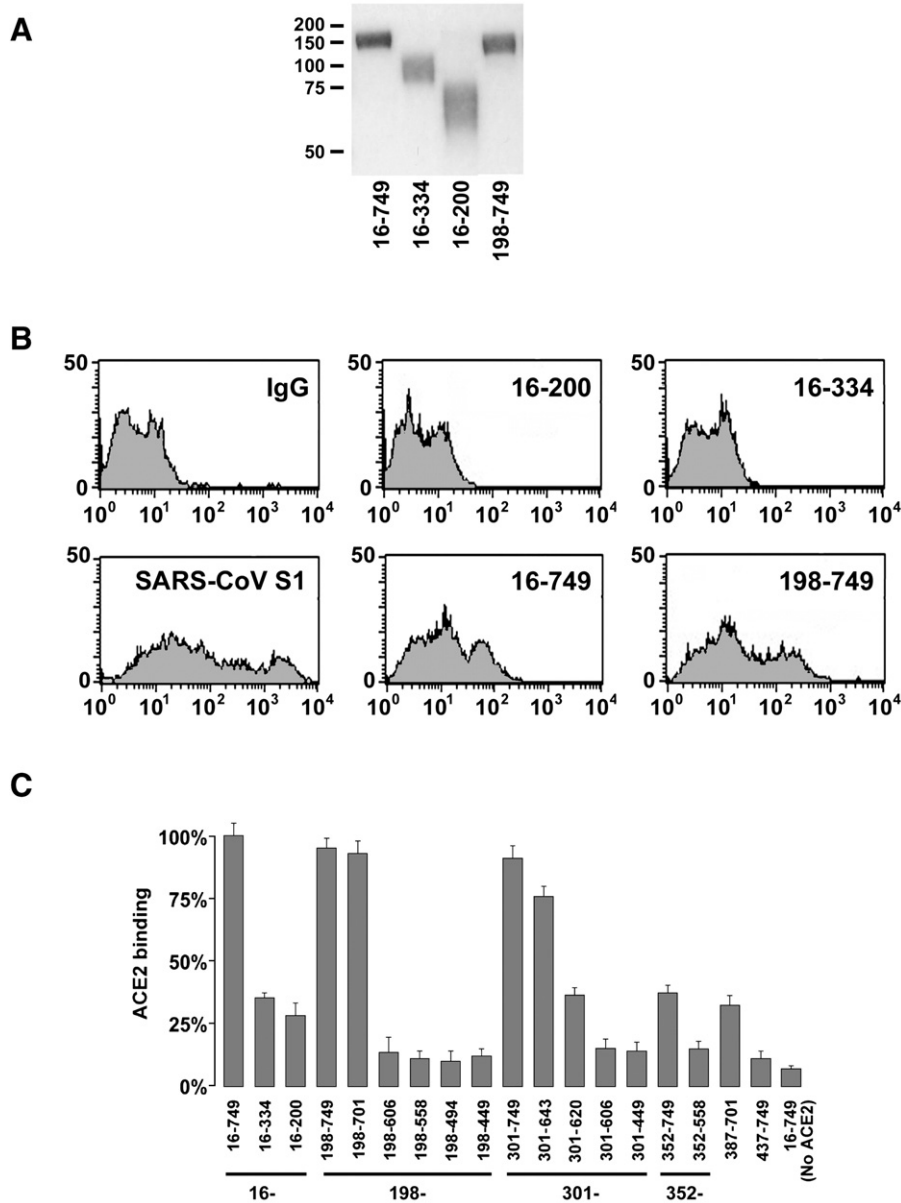


Fig. 1. Residues 301 through 643 of HCoV-NL63 spike protein contribute to ACE2 association. (A) Fusion proteins expressing the indicated fragments of the HCoV-NL63 S protein and the Fc region of human IgG1 were purified from transfected HEK293T cells. Expression of S1-Ig (residues 16–749) and smaller variants were normalized for expression by Coomassie staining. (B) 5  $\mu$ g/ml of human IgG, HCoV-NL63 S1-Ig (residues 16–749), or the indicated truncation variants thereof, or SARS-CoV S1-Ig was incubated with human-ACE2-expressing HEK293T cells. Cells were washed and ACE2 association analyzed by flow cytometry. Mean fluorescence intensity: IgG, 3.4; 16–200, 3.7; 16–334, 4.4; SARS-CoV S1, 79.3; 16–749, 18.2; 198–749, 20.5. (C) Experiment similar to that in panel B except that a wider range of truncation variants of HCoV-NL63 S1-Ig were assayed. Binding, measured as mean fluorescence intensity, is shown as percentage of binding observed with HCoV-NL63 S1-Ig.

#### *Some ACE2 residues contribute to both HCoV-NL63 and SARS-CoV S-protein association*

The ability of rat, palm civet, and human ACE2 to bind the SARS-CoV S protein has been compared previously (Li et al., 2004, 2005c). The SARS-CoV S protein binds rat ACE2 very inefficiently, whereas it binds palm civet ACE2 more efficiently than human ACE2 (Li et al., 2005c). We compared the ability of the HCoV-NL63 S protein to bind rat and palm civet ACE2 molecules (Fig. 3A). We also characterized two forms of palm civet ACE2, varying at residue 354. Wild-type palm civet

ACE2 bears an aspartic acid at this position, whereas the ACE2 of most animals, including human, rat, mouse, cat, and dog express a glycine (Li et al., 2004, 2005c). Similar to what we have reported for SARS-CoV, HCoV-NL63 S1-Ig(301–749) did not bind rat ACE2 efficiently and bound the D354G form of palm civet ACE2 more efficiently than human ACE2. However, in striking contrast to the SARS-CoV S1, HCoV-NL63 S1-Ig (301–749) did not bind palm civet ACE2 with the native aspartic acid at residue 354. Therefore, residues in the immediate vicinity of glycine 354 likely contribute to HCoV-NL63 association.

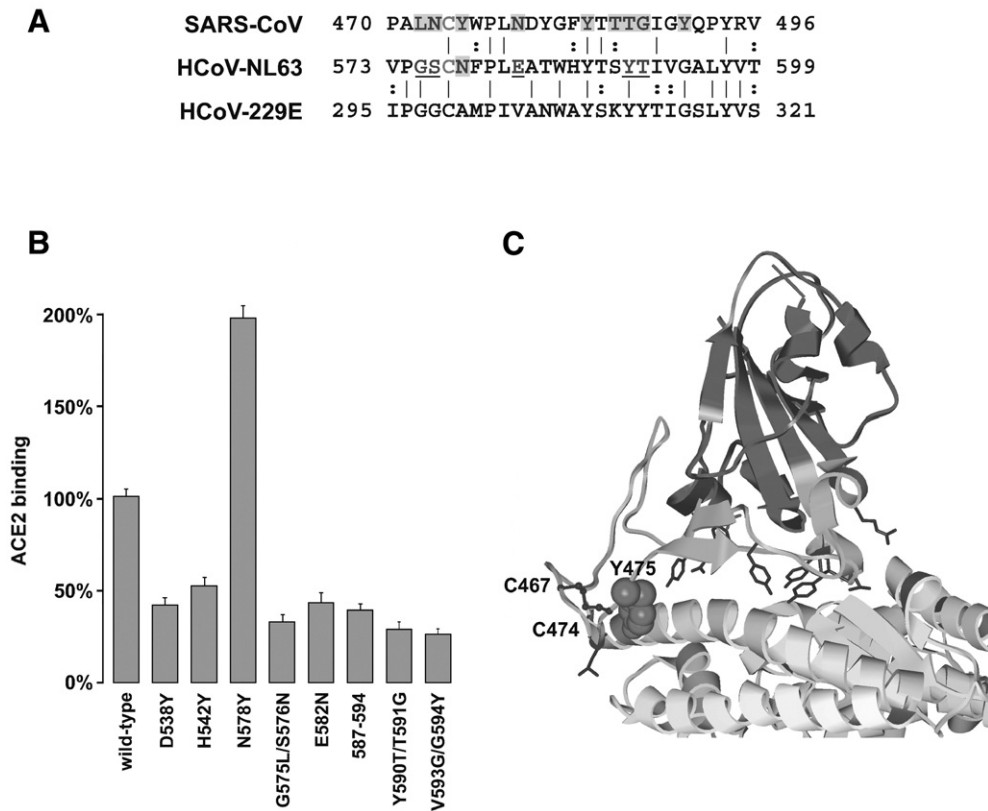


Fig. 2. Alteration of a putative HCoV-NL63 receptor-binding motif modulates ACE2 association. (A) Alignment of the SARS-CoV RBM with similar regions of HCoV-NL63 and HCoV-229E S proteins. SARS-CoV residues that directly contact ACE2 are highlighted, as is HCoV-NL63 S-protein asparagine 578, whose alteration to tyrosine enhances association with ACE2. HCoV-NL63 residues whose alteration decreases association with ACE2 are underlined. (B) Experiment similar to that of Fig. 1C except that HCoV-NL63 S1-Ig(301–749) variants altered in its putative receptor-binding motif are characterized. (C) The structure of the SARS-CoV RBD bound to ACE2 (white). The RBM is shown in light grey, and RBM residues in direct contact with ACE2 are shown in black. Disulfides forming a small loop, which may be similar to loops in HCoV-NL63 and HCoV-229E, are indicated. Tyrosine 475, adjacent to the C-terminal cysteine of this loop, is represented as spheres.

We further compared the ability of human ACE2 variants to bind the S1 domains of HCoV-NL63 and SARS-CoV. As we have previously reported, introduction of a glycosylated region of rat ACE2 (residues 82–84; denoted MYP/NFS) into the human protein modestly decreased binding of the SARS-CoV S1 (Li et al., 2005c). This modification similarly decreased HCoV-NL63 S1 binding (Fig. 3B). We also introduced into human ACE2 four residues from the palm civet receptor that resulted in loss of a glycosylation site in the human receptor (residues 90–93, denoted NLTV/DAKI). As we previously reported, this alteration enhanced SARS-CoV S1 association (Li et al., 2005c). Again, the HCoV-NL63 S protein followed the same pattern. However, introduction of an aspartic acid at human ACE2 residue 354 had only a modest effect on SARS-CoV S1 association, whereas it completely abolished association with HCoV-NL63 S1 (Fig. 3B). Thus residue 354 modulates HCoV-NL63 S-protein association with both human and palm civet ACE2 (Figs. 3A and B). As shown in Fig. 3C, the ability of these human ACE2 variants to bind HCoV-NL63 and SARS-CoV S1 was reflected in their ability to support infection mediated by the S proteins of these viruses.

We also characterized a series of human ACE2 variants previously described (Li et al., 2005c) for their ability to bind SARS-CoV S1 and support SARS-CoV infection (Fig. 3D). Each

variant expressed efficiently, as indicated by antibody recognition of an N-terminal tag. Several variants that less efficiently bound the SARS-CoV S1 domain also less efficiently bound HCoV-NL63 S1-Ig(301–749). Specifically, alteration of tyrosine 41, lysine 353, aspartic acid 355, and arginine 393 interfered with the ability of S1-Ig(301–749) to bind, and more modestly, to support HCoV-NL63 S-protein-mediated infection. We also characterized a number of alterations in solvent-exposed ACE2 residues previously shown to not interfere with SARS-CoV S1-Ig association. None of these alterations interfered with HCoV-NL63 S1-Ig(301–749) binding or entry mediated by the HCoV-NL63 S protein (Supplementary Table 1). Collectively, these data indicate that the SARS-CoV and HCoV-NL63 S1 domains bind regions of ACE2 that largely overlap. The observation that the G354D alteration strongly interferes with binding of the HCoV-NL63, but not the SARS-CoV, S1 domain also supports this assertion: residue 354 is immediately adjacent to lysine 353, a residue critical for the association of the SARS-CoV S protein to ACE2.

#### *The SARS-CoV RBD inhibits infection mediated by the HCoV-NL63 S protein*

Finally, we assayed the ability of the SARS-CoV RBD to inhibit infection mediated by the SARS-CoV and HCoV-NL63 S



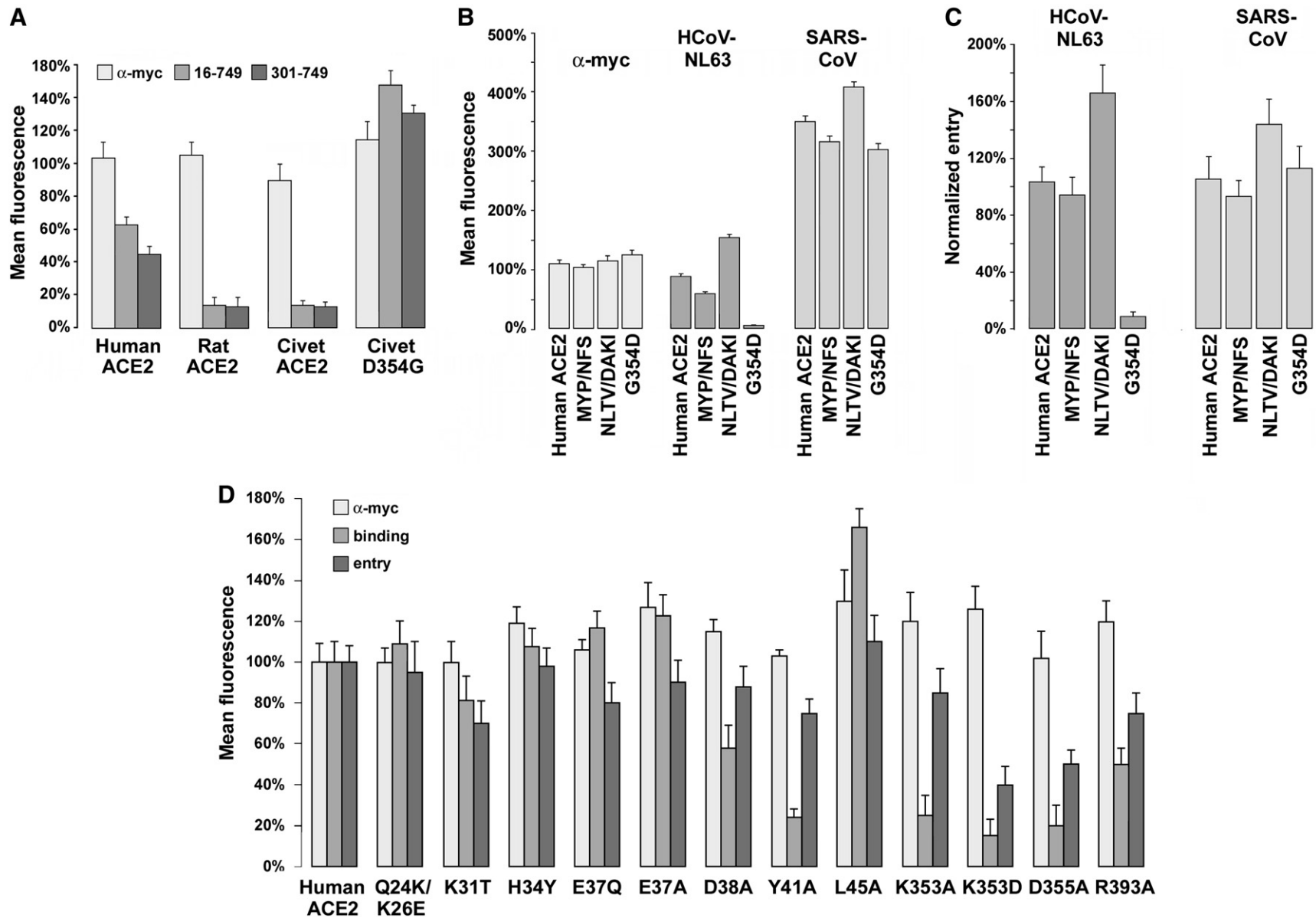


Fig. 3. HCoV-NL63 and SARS-CoV S proteins bind similar sites on human ACE2. (A) HEK293T cells expressing N-terminally tagged forms of human, rat, or two variants of palm civet ACE2 proteins incubated with anti-tag antibody, or HCoV-NL63 S1-Ig(16–749) or S1-Ig(301–749). Cells were washed and analyzed by flow cytometry. Results are indicated as the percentage of mean fluorescence intensity observed with cells expressing human ACE2 and incubated with anti-tag antibody. (B) An experiment similar to (A) except that human ACE2, and three indicated variants thereof, was analyzed for binding to anti-tag antibody, HCoV-NL63 S1-Ig(301–749), and SARS-CoV S1-Ig. (C) Lentiviruses expressing luciferase and pseudotyped with the S proteins of HCoV-NL63 or SARS-CoV were incubated with cells transfected with human ACE2 or the indicated human ACE2 variants, as previously described (Li et al., 2005c; Sui et al., 2005). Entry, as measured by luciferase activity, is shown as percentage observed for wild-type human ACE2. (D) Experiments similar to those of (B) and (C) except that human ACE2 variants proximal to the SARS-CoV binding site are characterized for expression, HCoV-NL63 S1-Ig(301–749) binding, and HCoV-NL63 S-protein-mediated infection. See Supplemental data for results of experiments characterizing variants that do not contact the SARS-CoV RBD.

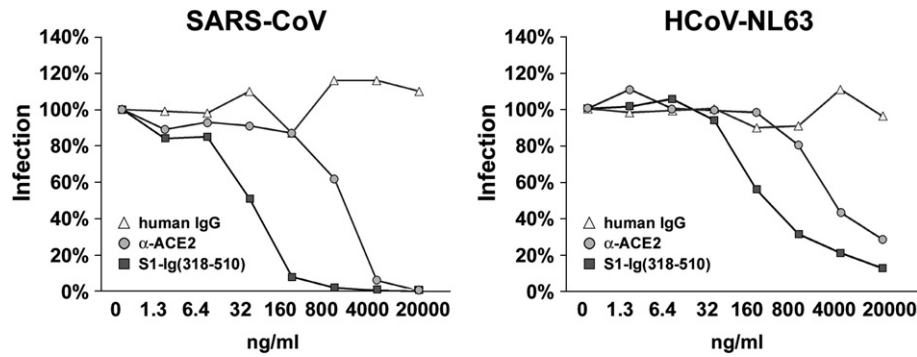


Fig. 4. The SARS-CoV RBD inhibits HCoV-NL63 S-protein-mediated infection. Lentiviruses expressing luciferase and pseudotyped with the S proteins of SARS-CoV or HCoV-NL63 were incubated with ACE2-expressing HEK293T cells in the presence of the indicated concentrations of affinity purified goat anti-ACE2 antibody (Li et al., 2003), SARS-CoV RBD-Ig, or human IgG. Infection is expressed as a percentage of luciferase activity observed in the absence of inhibitor.

proteins (Fig. 4). The SARS-CoV RBD specifically inhibited HCoV-NL63 S-protein-mediated infection. As previously reported, this RBD also blocked entry mediated by the SARS-CoV S protein (Wong et al., 2004). Consistent with data from Fig. 3, this observation indicates that the HCoV-NL63 S protein utilizes ACE2 residues occluded by the SARS-CoV RBD. The reduced ability of the SARS-CoV RBD to inhibit entry mediated by the HCoV-NL63 S protein, relative to the SARS-CoV S protein, may reflect other HCoV-NL63 binding sites on ACE2 or a greater role for other cellular proteins. Of note, neither the lower affinity HCoV-NL63 S1(301–749)-Ig nor a N578Y variant inhibited entry mediated by the S proteins of HCoV-NL63 or SARS-CoV at the concentrations used in Fig. 4 (not shown). Fig. 5 summarizes our observations with a “virus-eye” view of human ACE2, highlighting residues critical to the S proteins of both viruses.

## Discussion

HCoV-NL63 is closely related to HCoV-229E and a number of other group I viruses found in animals (Fouchier et al., 2004; Hofmann et al., 2005; Pyrc et al., 2004; van der Hoek et al., 2004). Most group I coronaviruses utilize the cellular receptor CD13 (Holmes et al., 1993). Surprisingly, HCoV-NL63 utilizes ACE2, the same receptor used by SARS-CoV (Hofmann and Pohlmann, 2004; Hofmann et al., 2005; Smith et al., 2006). Although the S1 domains of HCoV-NL63 and SARS-CoV are quite different, the region of SARS-CoV that directly contacts ACE2, the RBM, bears some similarity to a region present in most group I coronaviruses including HCoV-NL63 (Li et al., 2006). This region is not present in group II coronaviruses other than SARS-CoV. Interestingly, the S1 proteins of SARS-CoV-like viruses found in bats lack much of this region (Lau et al., 2005; Li et al., 2005b, 2006). It is not established whether this region has been deleted in these bat viruses, or whether SARS-CoV acquired this region, perhaps through convergent evolution or recombination with a group I relative of HCoV-NL63. Data here are somewhat consistent with acquisition of the RBM during SARS-CoV evolution. We have shown that perturbations of the RBM-like region in HCoV-NL63 S protein interfere with its ability to mediate viral entry. Interestingly, when a residue found in the RBM of SARS-CoV was introduced into a weakly homologous region of the HCoV-NL63 S protein, the affinity of

this S protein for ACE2 was substantially enhanced, although not to the level observed for the SARS-CoV S protein.

We have also shown that HCoV-NL63 entry is sensitive to some changes in ACE2 that interfere with or are adjacent to residues critical for SARS-CoV entry. In addition, the SARS-CoV RBD can inhibit HCoV-NL63 S-protein-mediated infection. These data indicate that the SARS-CoV and HCoV-NL63 binding sites on ACE2 overlap. In contrast, Hoffman et al., using a different panel of ACE2 variants, tentatively concluded that these viruses have distinct binding sites because they did not identify variants that interfered both with SARS-CoV and HCoV-NL63 entry (Hofmann et al., 2006). Here we shown that Y41A and K353D ACE2 variants, previously shown to limit SARS-CoV entry (Li et al., 2005c) and contact the SARS-CoV RBD, also limited HCoV-NL63 infection. In addition, two variants in a loop formed by ACE2  $\beta$ 4 and  $\beta$ 5 strands, D355A and especially G354D, adjacent to lysine 353, substantially

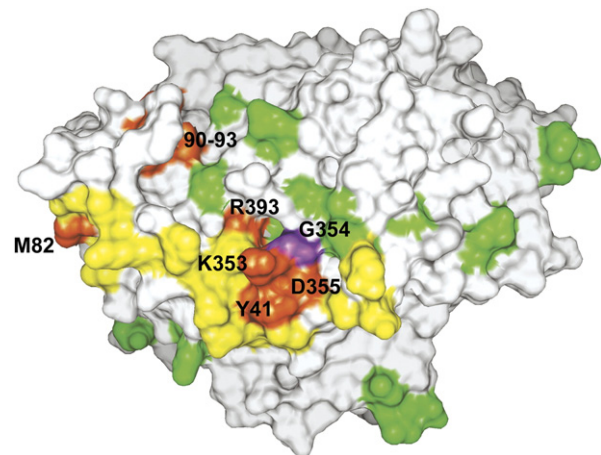


Fig. 5. ACE2 residues critical to HCoV-NL63 S1-Ig association. A “virus-eye” view of the human ACE2 surface is shown. Residues in contact with the SARS-CoV RBD are indicated in yellow and orange. Residues whose alteration affects HCoV-CoV S1 binding were colored orange. Glycine 354, whose alteration to aspartic acid abolishes HCoV-NL63 binding, but not that of SARS-CoV, is shown in purple. Residues whose alteration does not alter ACE2 association with either S protein are shown in green. Some green residues listed in Supplementary Table 1 are not visible in this view.

interfered with HCoV-NL63 infection. Use of a similar region on ACE2 may be a result of functional constraints on viral entry, or it may reflect a common origin of the receptor-binding motifs of these divergent viruses.

The observation that HCoV-NL63 and SARS-CoV bind a similar region on ACE2 also has a practical implication: small molecules that bind ACE2 and inhibit SARS-CoV infection will also likely inhibit replication of HCoV-NL63. Although the HCoV-NL63 virus circulating in the human population is only modestly pathogenic in most cases, an inhibitor may be useful in treating the subset of serious cases or more pathogenic forms of the virus.

## Methods

### *HCoV-NL63 and SARS-CoV S protein and ACE2 variants*

Plasmids encoding a codon-optimized form of the SARS-CoV S-protein, TOR2 isolate (accession number AY274119), and of the HCoV-NL63 S protein (AY567487) have been previously described (Huang et al., 2006; Li et al., 2003; Moore et al., 2004). Plasmids encoding the S1 domain (residues 12–672) and the receptor-binding domain (residues 318–510) of the TOR2 S protein, fused to the Fc domain of human IgG1 (S1-Ig and RBD-Ig, respectively), have been previously described (Li et al., 2003; Wong et al., 2004). Plasmids encoding the S1 domain of HCoV-NL63 were generated by inserting codon-optimized sequence encoding residues 16–749 into the identical vector used to express SARS-CoV S1-Ig between the unique *NheI* and *BamHI* sites. Sequences encoding truncation variants of HCoV-NL63 S1-Ig were generated by PCR and ligated at the same sites of the same vector. Variants of HCoV-NL63 S1-Ig and S1-Ig(301–749) were generated by mutagenesis using the QuikChange method (Invitrogen). Human, rat, and palm-civet ACE2 molecules, and variants thereof, encoding an amino-terminal tag from c-myc have been previously described (Li et al., 2004, 2005c).

### *Binding assays*

Association of HCoV-NL63 S1-Ig or variants with ACE2 variants was determined by flow cytometry. Flow cytometry using ACE2-expressing cells has been previously described (Li et al., 2003; Wong et al., 2004). Briefly, HEK293T cells were transfected with a plasmid encoding ACE2 variants, or with vector alone. Two days post-transfection, cells were detached in PBS/5 mM EDTA and washed with PBS/0.5% BSA. S1-Ig, or variants thereof, or the anti-tag antibody 9E10, was added to  $5 \times 10^5$  cells, and the mixture was incubated on ice for 1 h. Cells were washed three times with PBS/0.5% BSA then incubated for 30 min on ice with anti-human IgG FITC conjugate (Sigma). Cells were again washed with PBS/0.5% BSA and analyzed.

### *Infection with S-protein-pseudotyped lentiviruses*

Infection was assayed with a lentivirus expressing a luciferase reporter gene and pseudotyped with SARS-CoV or

HCoV-NL63 S protein, as previously described (Sui et al., 2005). Briefly, HEK293T cells were cotransfected with plasmid encoding full-length S proteins, a plasmid (pCMV $\Delta$ R8.2) encoding HIV-1 Gag-Pol, and a plasmid (pHIV-Luc) encoding the firefly luciferase reporter gene under the control of the HIV-1 long terminal repeat. Two days post-transfection, viral supernatants were harvested and 3  $\mu$ l of S-protein-pseudotyped virus was used for infection of 6000 ACE2-expressing HEK293T cells in a 96-well plate, with varying concentrations of SARS-CoV S1-Ig(318–510), human IgG, or an affinity-purified anti-ACE2 polyclonal antibody (R&D Systems). Infection efficiency was quantified by measuring luciferase activity in target cells with an EG&G Berthold Microplate Luminometer LB 96V.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.04.035.

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