Chapter 3.1

THE SARS CORONAVIRUS RECEPTOR ACE2 A POTENTIAL TARGET FOR ANTIVIRAL THERAPY

J. H. KUHN^{1,2}, S. R. RADOSHITZKY¹, W. LI¹, S. KEE WONG¹,
H. CHOE³ and M. FARZAN¹

¹*Department of Microbiology and Molecular Genetics, Harvard Medical School, New England Primate Research Center, Southborough, MA, USA;* ²*Department of Biology, Chemistry, Pharmacy, Freie Universität Berlin, Berlin, Germany;* ³*Department of Pediatrics, Children's Hospital, Harvard Medical School, Boston, MA, USA*

Abstract: In recent years, coronaviruses have received increasing attention from clinical infectious disease experts. The discovery of a coronavirus as the etiological agent of severe acute respiratory syndrome (SARS-CoV) and the identification of another coronavirus that causes upper respiratory tract diseases in children (HCoV-NL63) emphasized the importance of improving our understanding of these agents. The identification of angiotensin-converting enzyme 2 (ACE2), the cell-surface receptor for both SARS-CoV and HCoV-NL63, has opened the field for the development of new treatments and prophylactics. In this review, we describe the discovery of this coronavirus receptor and its interaction with coronaviral surface proteins in light of viral entry processes and host tropism.

1. INTRODUCTION

1.1 Coronaviruses

The viral order *Nidovirales* groups numerous viruses of vertebrates and invertebrates that share common characteristics such as genomic organization, transcription and replication strategies, and disease manifestations. Many of these viruses cause acute and chronic upper

respiratory gastrointestinal, hepatic, and neurological diseases in both humans and animals (Enjuanes *et al.*, 2000). The nidoviruses are compartmentalized into the three viral families *Arteriviridae*, *Coronaviridae*, and *Roniviridae*. Nidoviral pathogens that infect humans belong exclusively to the family *Coronaviridae*, which contains the two genera *Coronavirus* and *Torovirus* (Enjuanes *et al.*, 2000).

Coronaviruses *sensu stricto* (viruses belonging to the genus *Coronavirus* rather than all members of the family *Coronaviridae*) are spherical to slightly pleomorphic viruses with a diameter of 100-160nm. The virions contain one copy of a 20-33kb-long capped and polyadenylated single-stranded RNA of positive polarity, which is helically encapsidated by nucleocapsid (N) proteins (Enjuanes *et al.*, 2000). The filamentous ribonucleocapsids are surrounded by matrix (M) proteins, which form icosahedral cores. These cores are wrapped in envelopes formed during coronaviral budding from a host cell. The membranes contain distinct, club- or petal-shaped protrusions, identified as spike (S) proteins. It is those proteins that give the virions a crown-like appearance (Latin: *coronae*) in electron microscopic images (Enjuanes *et al.*, 2000). S proteins are the major antigenic determinants of coronaviruses (Daniel *et al.*, 1994; Gallagher *et al.*, 2001; Koo *et al.*, 1999; Moore *et al.*, 1997; Song *et al.*, 1998).

Coronaviruses attach to cell-surface receptors via their S proteins, which then mediate fusion with the host-cell membrane. Subsequently, a nested set of subgenomic RNAs is produced. Genome replication occurs in the cytoplasm and results in high-frequency recombination. After replication and maturation, coronaviruses bud from the endoplasmic reticulum-Golgi intermediate compartment (Enjuanes *et al.*, 2000).

Three distinct genetic and serological groups of coronaviruses are acknowledged, but the grouping is undergoing revision (Gonzalez *et al.*, 2003). Several coronaviruses are known to cause disease in humans. Human coronavirus 229E (HCoV-229E), a group 1 virus, and human coronavirus OC43 (HCoV-OC43), a group 2 virus, cause mild upper respiratory infections that result in self-resolving common colds in otherwise healthy individuals, or severe pneumonia in immunocompromised people (Denison, 1999; Pene *et al.*, 2003). Human coronavirus NL63 (HCoV-NL63; also referred to as HCoV-NH and HCoV-NL) has recently been identified as a group 1 virus causing conjunctivitis and sometimes serious respiratory infections in children (Esper *et al.*, 2005b; Fouchier *et al.*, 2004; van der Hoek *et al.*, 2004). Epidemiological analyses suggest this virus to be associated with Kawasaki disease (Esper *et al.*, 2005a). Another group 2 coronavirus (CoV-HKU1) was recently isolated from a 71-year old man with pneumonia (Woo *et al.*, 2005). Several more pathogenic human

coronaviruses (B814, HCoV-OC16, HCoV-OC37, and HCoV-OC48) await characterization (McIntosh, 2005).

1.2 Severe acute respiratory syndrome virus

Severe acute respiratory syndrome (SARS) was first described in November of 2002, when inhabitants of Guangdong Province, People's Republic of China, presented with an influenza-like illness that began with dyspnea, myalgia, and pyrexia, and was often followed by acute pneumonia, respiratory failure, and death (Peiris *et al.*, 2003). The novel disease was transmitted via droplets and fomites and through direct contact of patients with uninfected individuals (Olsen *et al.*, 2003; Yu *et al.*, 2004). The outbreak spread all over Asia, Europe, and North America. At the end of the mini-pandemic in July 2003, a total of 8,096 cases had been recorded, of which 774 (9.6%) had died (Cherry, 2004). A novel coronavirus, severe acute respiratory syndrome virus (SARS-CoV; species *Severe acute respiratory syndrome coronavirus*) was identified as the etiologic agent (Drosten *et al.*, 2003; Fouchier *et al.*, 2003; Ksiazek *et al.*, 2003; Kuiken *et al.*, 2003; Mayo, 2002). Molecular analyses demonstrated that SARS-CoV clusters with group 2 coronaviruses (Gibbs *et al.*, 2004; Gorbalenya *et al.*, 2004).

SARS reemerged in Guangdong Province in 2003-2004, when it infected four individuals, all of which recovered (Fleck, 2004; Song *et al.*, 2005). All other cases recorded to date trace back to accidental laboratory infections. One and two nonfatal cases, respectively, were recorded in laboratories in the Republic of Singapore (Lim *et al.*, 2004) and Taiwan (Normile, 2004) in 2003. The last cases occurred in 2004 in laboratories in Anhui Province (nine cases including one fatality) and in Beijing (two cases), People's Republic of China.

The natural reservoir of SARS-CoV remains elusive. Chinese ferret-badgers (*Melogale moschata*), Himalayan palm civets (*Paguma larvata*), and racoon dogs (*Nyctereutes procyonoides*) have been suggested as host candidates, because they carry SARS-CoV-like viruses (Guan *et al.*, 2003). Domestic cats (*Felis domesticus*), ferrets (*Mustela putorius furo*), and Himalayan palm civets have been proposed as potential carriers because they are susceptible to SARS-CoV infection and because they can transmit the agent (Martina *et al.*, 2003; Wu *et al.*, 2005). BALB/c mice (Subbarao *et al.*, 2004; Wentworth *et al.*, 2004), hamsters (Roberts *et al.*, 2005), and cynomolgus and rhesus macaques (Rowe *et al.*, 2004) are currently being used as animal models for SARS-CoV infection.

As of today, vaccines have not been approved for the prevention of human SARS-CoV infections, although several candidates are clinically evaluated.

Sera obtained from SARS survivors contain high titers of SARS-CoV-neutralizing antibodies. SARS patients were reported to clinically improve after the administration of such reconvalescent sera (Li *et al.*, 2003a; Pearson *et al.*, 2003). Neutralizing antibodies, administered to BALB/c mice intraperitoneally one day before intranasal challenge with 10^4 50% tissue culture infective doses (TCID₅₀) of the SARS-CoV Urbani strain, reduced viral load by four orders of magnitude (Sui *et al.*, 2005). These data suggest that antibodies could be used to prevent and treat SARS, and also that the development of a SARS vaccine is plausible. Promising results in vaccine development have been obtained with inactivated whole virus (He *et al.*, 2004b; Takasuka *et al.*, 2004), recombinant N or S protein administered either as a DNA vaccine (Yang *et al.*, 2004; Zhao *et al.*, 2005), via replication-deficient adenoviruses (Zakhartchouk *et al.*, 2005) or *Lactococcus lactis* (Pei *et al.*, 2005), as well as via S protein-expressing vaccinia viruses (Bisht *et al.*, 2004; Chen *et al.*, 2005). In all these cases, development of neutralizing antibodies was reported after immunization, and in the majority, these vaccines protected mice from challenge with infectious SARS-CoV. However, mice do not develop a SARS-like disease after viral infection. SARS-CoV infection only leads to high levels of replication in their respiratory tracts, and virus is cleared within seven days post infection (Subbarao *et al.*, 2004). Hence, it remains unclear whether the vaccine candidates tested would also be efficacious in primates or, later, in humans. Studies in nonhuman primates have been sparse, but at least one group achieved protection of African green monkeys (*Chlorocebus aethiops*) against SARS-CoV challenge after vaccination with an attenuated parainfluenza virus expressing SARS-CoV S protein (Bukreyev *et al.*, 2004).

Obvious targets for antiviral agents include the SARS-CoV protease, mRNA cap-1 methyl-transferase, NTPase/helicase, and the transcriptase-replicase complex. However, with the exception of interferons, which have been shown effective in inhibiting SARS-CoV replication in tissue cultures, no clinically approved antiviral drug could be identified for the treatment of SARS so far (Tan *et al.*, 2004).

1.3 Coronaviral spike proteins and host-cell receptors

The host spectrum and virulence of a specific coronavirus is largely determined by its S protein (Kuo *et al.*, 2000; Phillips *et al.*, 1999; Sanchez *et al.*, 1999). Coronaviral S proteins measure 12-24 nm in length. They are spaced widely apart, and are evenly distributed all over the surface of the

coronavirion (Enjuanes *et al.*, 2000). They are type I transmembrane and class I fusion proteins (Bosch *et al.*, 2003; Gallagher, 2001), and consist of distinct N-terminal (S1) and C-terminal (S2) domains, which mediate receptor binding and virus-cell fusion, respectively (Gallagher, 2001). Upon binding of the cell-surface receptor, a conformational change of the S1 domain induces the exposure of a fusion peptide embedded in the S2 domain, which then induces the reorganization of S2's unusually large heptad repeats into coiled-coils. This extreme conformation switch forces the virion membrane into close apposition to the cell membrane and allows fusion and hence penetration of the viral core into the cell (Colman *et al.*, 2003; Dimitrov, 2004; Lai *et al.*, 1997).

Only few coronavirus cell-surface receptors have been identified. Aminopeptidase N (APN, CD13) was shown to be the receptor for canine coronavirus, feline infectious peritonitis virus, HCoV-229E, porcine epidemic diarrhea virus, and porcine transmissible gastroenteritis virus, all of which are group 1 coronaviruses (Delmas *et al.*, 1992; Oh *et al.*, 2003; Yeager *et al.*, 1992). Members of the pleiotropic family of carcinoembryonic antigen-cell adhesion molecules were identified as receptors for the group 2 pathogen murine hepatitis virus (Williams *et al.*, 1991), whereas bovine group 2 coronaviruses bind to 9-*O*-acetylated sialic acids (Holmes *et al.*, 1994).

2. IDENTIFICATION OF ACE2 AS THE RECEPTOR OF SARS CORONAVIRUS AND HUMAN CORONAVIRUS NL63

Full-length and partial S proteins of different SARS-CoV isolates were cloned, expressed, and characterized by several research groups only months after the discovery of the virus (Babcock *et al.*, 2004; Bisht *et al.*, 2004; Li *et al.*, 2003b; Simmons *et al.*, 2004; Wong *et al.*, 2004; Xiao *et al.*, 2003). SARS-CoV S protein was found to have a molecular weight of about 170-200 kD in PAGE analyses. The protein was suggested to undergo posttranslational modifications since the determined weight was much higher than the molecular weight predicted from the amino acid sequence (Bisht *et al.*, 2004; Xiao *et al.*, 2003). SARS-CoV S protein is a typical class I fusion protein. However, there is little (20-27%) similarity between the amino acid sequence of S protein of SARS-CoV and those of other coronaviral S proteins (Rota *et al.*, 2003). Furthermore, in contrast to most other S proteins, SARS-CoV S protein is not cleaved by a host cell protease into S1 and S2 subunits, although domains corresponding to S1 and S2 have been identified (Marra *et al.*, 2003; Rota *et al.*, 2003; Xiao *et al.*, 2003).

SARS-CoV S protein alone is sufficient to mediate virus-cell fusion. The SARS-CoV S1 domain was identified as the receptor-binding region of the attachment protein, whereas the S2 domain was found to mediate fusion with the host cell membrane (Li *et al.*, 2003b; Xiao *et al.*, 2003). However, it was impossible to predict which cell-surface receptor SARS-CoV might utilize to fuse with host cells, in part because of the limited sequence homology between the SARS-CoV S1 domain and the S1 proteins of other coronaviruses. In fact, the observed severity and lethality of SARS-CoV infections and the elucidated limited S1 sequence homology with other S proteins suggested a receptor different from all other known coronavirus receptors.

Our laboratory employed a straight-forward approach, coimmunoprecipitation, to identify the SARS-CoV cell surface receptor (Li *et al.*, 2003b). We first created a codon-optimized gene encoding the SARS-CoV Urbani strain S1 domain (amino acid residues 12-672) fused to the Fc domain of human IgG1 (S1-Ig). Codon optimization resulted in markedly enhanced expression levels compared to wild-type S protein (Moore *et al.*, 2004). The construct bound to the surface of African green monkey kidney (Vero E6) cells, which are highly susceptible to SARS-CoV infection, with high affinity, but not to human embryonic kidney (HEK 293T) cells, in which SARS-CoV replicates poorly (Li *et al.*, 2003b). Encouraged by this observation, we lysed metabolically labelled Vero E6 cells with a detergent (0.3% *n*-decyl- β -D-maltopyranoside in PBS), and incubated the lysate with SARS-CoV S1-Ig and Sepharose-Protein A beads. Bound proteins were separated by PAGE, and individual bands, which were not present in control reactions, were analyzed by trypsin digestion and mass spectrometry. We identified a promising SARS-CoV receptor candidate, the 110kD human angiotensin-converting enzyme 2 (ACE2) (Li *et al.*, 2003b). A soluble form of ACE2, but not of the closely related ACE1, blocked binding of S1-Ig to the surface of Vero E6 cells. Antibodies to ACE2, but not those to ACE1, blocked SARS-CoV (strain Urbani) replication in Vero E6 cells. SARS-CoV replicated more efficiently in HEK 293T cells after ACE2 transfection. Last, we demonstrated syncytia formation between S protein-expressing HEK 293T cells and HEK 293T cells expressing ACE2 (Li *et al.*, 2003b), suggesting that the syncytia observed in SARS-CoV-infected Vero E6 cells, nonhuman primates, and patients (Drosten *et al.*, 2003; Fouchier *et al.*, 2003; Kuiken *et al.*, 2003) develop through S protein-ACE2 interactions. Commercially available antibodies targeting ACE2 blocked syncytia formation (Li *et al.*, 2003b). Stably ACE2-expressing HEK 293T cells were more transducible with S protein-pseudotyped simian immunodeficiency virus than VeroE6 cells (Moore *et al.*, 2004), which is consistent with the idea that the abundance of ACE2 on the cell surface determines the

efficiency of SARS-CoV infection. Others have identified a recombinant human single-chain variable region fragment (scFV) against the SARS-CoV (strain TOR2) S1 domain from nonimmune human antibody libraries. This scFV (named 80R scFV) not only inhibited the formation of syncytia between HEK 293T cells expressing ACE2 and HEK 293T cells expressing the S protein, but also efficiently neutralized infection of Vero E6 cells with the SARS-CoV Urbani strain. 80R scFV competed with a soluble form of ACE2 for association with the S1 domain, and was found to bind S1 with high affinity ($K_d=32.3$ nM) in plasmon resonance studies. A human IgG1 form of 80R scFV (80R) bound S1 with higher affinity ($K_d=1.59$ nM) and neutralized the Urbani strain of SARS-CoV with a 20-fold higher efficiency than 80R scFV (Sui *et al.*, 2004). ACE2 was also independently identified as a SARS-CoV receptor by another group, which transduced HeLa cells with a retrovirus cDNA library from Vero E6 cells, followed by flow cytometry to select transduced cells that bound to purified S protein (residues 14-502) (Wang *et al.*, 2004).

ACE2, a carboxy-metalloprotease not related to any other coronavirus receptor but distantly related to ACE, was discovered in 2000 (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000). The enzyme is a type I transmembrane protein with a single metalloprotease active site with a HEXXH zinc binding motif. (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000). The physiological function of ACE2 remains enigmatic. The enzyme has been shown to cleave a variety of regulatory peptides *in vitro*, among them angiotensin I and II, des-Arg-bradykinin, kinetensin, and neurotensin (Donoghue *et al.*, 2000; Vickers *et al.*, 2002). Some cleavage products have been shown to be potent vasodilators with antidiuretic effects. This finding gave rise to the theory that ACE2 counterbalances the actions of ACE1, which mediates vasoconstriction (Yagil *et al.*, 2003). Furthermore, targeted disruption of ACE2 in mice resulted in severe cardiac contractility defects (Crackower *et al.*, 2002).

Despite high expression levels of ACE2, only low levels of SARS-CoV replication were found in cardiac tissue (Farcas *et al.*, 2005). This finding suggested that another receptor or a coreceptor is necessary for successful cellular infection with SARS-CoV. Indeed, CD209L (DC-SIGNR, L-SIGN) has been identified as a molecule that can facilitate SARS-CoV infection (Jeffers *et al.*, 2004; Marzi *et al.*, 2004). However, the tissue distribution of ACE2 matches the tissue tropism of SARS-CoV. For example, high levels of ACE2 expression were detected in the gastrointestinal tract, kidneys, and lungs (Ding *et al.*, 2004; Donoghue *et al.*, 2000; Hamming *et al.*, 2004; Harmer *et al.*, 2002), all of which are targets of SARS-CoV (Hamming *et al.*, 2004). Others have shown that the level of ACE2 expression in different cell lines correlates with their susceptibility for SARS-CoV infection

(Hattermann *et al.*, 2005; Hofmann *et al.*, 2004) or their susceptibility for transduction with lentiviral pseudotypes carrying SARS-CoV S proteins (Nie *et al.*, 2004). Transfection of ACE2 into SARS-CoV-refractory cell lines conferred susceptibility to infection (Mossel *et al.*, 2005).

Taken together, these results imply that ACE2 is the major cell-surface receptor for SARS-CoV. They also suggest that tissues, which express ACE2 but are rather refractory to SARS-CoV infection, limit virus replication at a step following the interaction of the S protein with ACE2.

We and others also determined the receptor-binding domain (RBD) of SARS-CoV S1 (Babcock *et al.*, 2004; Wong *et al.*, 2004), which consists of only 193 amino acids (S1 residues 318-510; see Figure 1).

Figure 1: Receptor-binding domains of coronaviruses. Representation of the S proteins of SARS-CoV, HCoV-229E, HCoV-NL63 (NL63), and mouse hepatitis virus (MHV), aligned by their S2 domains. Dark gray indicates leader and transmembrane sequences. Light gray indicates receptor-binding domain. Future experiments may define the receptor-binding domain of NL63 more precisely than what is indicated. The receptor-binding domain of SARS-CoV is shown with N-glycosylation sites (small circles) and cysteines indicated. Residues that are critical to the high affinity interaction of SARS-CoV with human ACE2 (asparagine 479 and threonine 487) are shown as white bars.

An IgG1 Fc fusion protein (RBD-Ig) bound ACE2 more efficiently than full-length S1-Ig (Wong *et al.*, 2004). At least two amino acid residues (glutamic acid 452 and aspartic acid 454) are crucial for S1-ACE2 interaction (Wong *et al.*, 2004). In HEK 293T cells stably expressing ACE2, RBD-Ig blocked transduction of cells with S protein-pseudotyped simian immunodeficiency virus at a lower concentration ($IC_{50}=10$ nM) than full-length S1-Ig ($IC_{50}=50$ nM) (Wong *et al.*, 2004). RBD-Ig in combination with Freund's complete adjuvant induced strong antibody responses in intradermally immunized NZW rabbits. These antibodies completely inhibited infection of Vero E6 cells with 100 TCID₅₀ SARS-CoV at a serum dilution

of 1:10,240, and blocked the interaction of S1 with commercially available ACE2 (He *et al.*, 2004a). Similarly, the immunization of mice and rabbits with inactivated SARS-CoV induced antibodies that specifically recognize the RBD, block S1-RBD interaction, and prevent cell transduction with S protein-pseudotyped HIV-1 (He *et al.*, 2004b). Attenuated modified vaccinia virus Ankara expressing the SARS-CoV HKU38849 strain S protein induced neutralizing antibodies in immunized BALB/c mice, rabbits, and Chinese rhesus macaques. The antibodies were demonstrated to recognize a neutralizing epitope consisting of S protein's amino acid residues 400-600. This strongly suggested that the RBD was targeted by the antibodies. In fact, RBD-Ig could absorb the majority of neutralizing antibodies after incubation with antibody-containing sera (Chen *et al.*, 2005). Finally, the conformationally sensitive epitope recognized by the potent SARS-CoV-neutralizing antibody 80R (Sui *et al.*, 2004) was identified in S protein's N-terminal amino acid residues 324-503, which overlap the RBD, and 80R scFV precipitated RBD-Ig as efficiently as protein A (Sui *et al.*, 2005). Combined, these results strongly support the idea that SARS-CoV neutralizing antibodies target the receptor-binding domain of S protein and that virus neutralization occurs mainly through the interruption of the S1-ACE2 interaction.

Deletion studies demonstrated that the cytoplasmic domain of ACE2 is not important for ACE2-S1 interaction. Additionally, soluble ACE2 consisting only of its ectodomain blocked transduction with S protein-pseudotyped HIV-1 (Hofmann *et al.*, 2004). We demonstrated that an enzymatically inactive ACE2 variant, which contains two asparagine residues in place of the active-site histidine residues (ACE2-NN-Ig), still binds S protein and facilitates entry of S protein-pseudotyped lentiviruses. This finding demonstrated that the proteolytic activity of ACE2 plays no role in SARS-CoV cell penetration (Moore *et al.*, 2004). Also, binding of the S protein to ACE2 did not alter ACE2's enzymatic activity (Li *et al.*, 2005; Moore *et al.*, 2004). Finally, we showed that purified ACE2-NN-Ig prevented HEK 293T cell transduction with S-protein-pseudotyped simian immunodeficiency virus ($IC_{50} = 2$ nM), and that it blocked infection of VeroE6 cells with infectious SARS-CoV (Moore *et al.*, 2004).

The SARS-CoV-neutralizing antibody 80R blocked HEK 293T cell transduction with HIV-1 pseudotypes carrying the SARS-CoV strain TOR2 or strain SZ3 S proteins, but did not prevent transduction with pseudotypes carrying the strain GD03T0013 S protein (Sui *et al.*, 2005). This observation implied that the RBDs differ in key residues and suggested the possibility that different RBDs have different affinities to ACE2 of different species. A comparison of the RBD amino acid sequences of reported distinct SARS-

SARS-CoV isolates and SARS-CoV-like viruses from Himalayan palm civets revealed only few amino acid changes (see Table 1) (Sui *et al.*, 2005).

Table 1. Amino acid changes in the receptor binding domains (RBDs) of the spike (S) proteins of various SARS-CoV or SARS-CoV-like isolates according to (Sui *et al.*, 2005)

SARS-CoV isolate	Origin	GenBank accession No.	Amino acid at position 344	Amino acid at position 360	Amino acid at position 472	Amino acid at position 479	Amino acid at position 480	Amino acid at position 487
GD01	Early phase 2002-2003	AY278489	R/K	F	L	N	D	T
TOR2	Middle/ Late phase 2002-2003	AY274119	K	F	L	N	D	T
SZ3 (SARS-CoV-like)	Himalayan palm civet	AY304486, AY881174	R	S	L	K	D	S
GD03T0013	2003-2004	AY525636	R	S	P	N	G	S

The tissue distribution of murine and rat ACE2 was found to be comparable to that of human ACE2 (Gembardt *et al.*, 2005; Komatsu *et al.*, 2002). However, we found that murine ACE2 bound less efficiently to the SARS-CoV S1 domain and supported S protein-mediated infection less efficiently than human ACE2. Likewise, HEK 293T cell syncytia formation occurred at much higher efficiency when human ACE2, rather than murine ACE2, was expressed. Murine NIH 3T3 cells expressing human ACE2 supported SARS-CoV replication at a level of one magnitude higher than cells expressing murine ACE2. Rat ACE2, on the other hand, did not support S protein-mediated infection nor HEK 293T cell syncytia formation. (Li *et al.*, 2004). Interestingly, the exchange of only four amino acid residues of rat ACE2 for the equivalent residues found in human ACE2 (residues 82-84 and 353) transformed rat ACE2 into an efficient receptor for the SARS-CoV S1 domain (Li *et al.*, 2005).

Figure 2: The S protein-binding site on human ACE2. (Upper panel) The crystal structure of ACE2 is shown, oriented with the C-terminal “collectrin” domain downward and viewed from the front of the cleft bearing the enzymatic active site. Residues of rat ACE2 whose alteration to the corresponding human residues converted rat ACE2 to an efficient SARS-CoV receptor are shown in dark gray spheres. Human ACE2 residues whose alteration substantially decreased S1-Ig association are shown in light gray spheres. Residues whose alteration did not affect S1-Ig association are shown in stick figure format. Low-resolution electron density associated with the collectrin domain is represented by a small β -sheet and α -helix at the base of the figure. (Lower panel) A view identical to that in Upper panel except that the molecule has been rotated 90 degrees about the horizontal axis to present a “virus-eye” view of ACE2.

The findings led us to hypothesize that the host tropism of SARS-CoV and SARS-CoV-like viruses, as well as the severity of induced disease in different species might be dependent on RBD and ACE2 mutations. Experiments from our laboratory support this hypothesis. As shown in Figure 2, we localized the S protein-binding domain of ACE2 to α -helix 1 and to a loop of β -sheet 5 (Li *et al.*, 2005), both of which are located afar from the enzyme's active site but within the catalytic domain (Dales *et al.*, 2002; Towler *et al.*, 2004). S proteins from the different SARS-CoV strains isolated during the 2002-2003 and the 2003-2004 outbreaks as well as S proteins from the SARS-CoV-like viruses isolated from Himalayan palm civets bound to Himalayan palm civet ACE2 efficiently. However, as shown in Figure 3, the latter two proteins bound to human ACE2 much less efficiently than the S protein of the virus causing the initial 2002-2003 outbreak (Li *et al.*, 2005). Exchanging specific residues of human ACE2 for those found in Himalayan palm civet ACE2 markedly enhanced binding of the S proteins of the 2003-2004 and Himalayan palm civet viruses. Likewise, exchanging residues 479 and 487 of these S protein's RBDs for residues found in the S protein of virus isolates from the 2002-2003 outbreak (see Table 1) increased the binding efficiency to human ACE2 (Li *et al.*, 2005). Collectively, these data suggest that the apparent lack of severity and human-to-human transmission associated with the small SARS outbreak in 2003-2004 is in part due to incomplete adaptation of the associated virus isolate to human ACE2, and that this adaptation is mediated mainly by two amino acid residues. These results also provide biochemical support for the hypothesis that the palm civet was a source of SARS-CoV.

The tissue tropism of human coronavirus NL63 (HCoV-NL63) and SARS-CoV seems to be identical. Recently, using HIV-1 pseudotypes, it was shown that HCoV-NL63 also uses ACE2 as a cell-entry receptor, albeit with lower affinity (Hofmann *et al.*, 2005). This finding is surprising because the spike proteins of the two viruses share no amino acid identity. Additionally, this finding suggests that SARS is caused by an unidentified virulence factor of SARS-CoV, which is not encoded by the relatively harmless HCoV-NL63 genome. Clearly, further studies of the HCoV-NL63 entry mechanism are necessary. (Hofmann *et al.*, 2005)

Figure 3: Differential association of three S proteins with human and palm-civet ACE2. (A) 293T cells were transfected with plasmids encoding human or palm-civet ACE2, radiolabeled, and lysed. ACE2 proteins were immunoprecipitated with an antibody, which recognizes a tag present at the carboxy-terminus of each receptor, or with S1-Ig variants containing the S1 domains of TOR2, GD, or SZ3 S proteins, and analyzed by SDS-PAGE. TOR2 SARS-CoV was isolated from humans infected during the 2002-2003 outbreak; GD, from a small outbreak in 2003-2004 which resulted in mild symptoms and no human-to-human transmission; and SZ3, from palm civets. (B) An experiment similar to that in (A) except that S-protein residues 318-510, comprising the receptor-binding domains (RBDs) of the indicated S proteins fused to the Fc domain of human IgG1 (RBD-Ig), were used to immunoprecipitate human or palm-civet ACE2. The bottom panel shows a Coomassie-stained SDS-PAGE gel of the individual RBDs used in this experiment.

3. CONCLUSIONS

During its first recognized appearance in 2002, SARS-CoV quickly spread globally among human populations, and caused a novel severe disease in more than 8,000 people with a lethality of almost 10% (Cherry, 2004). Only one natural outbreak of SARS has been recorded since. During this emergence, only four people were infected and all survived (Fleck, 2004). This gave rise to the notion that SARS-CoV has disappeared; that future outbreaks of SARS are not likely; and that research funds would be better spent on other pathogens. However, the natural reservoir of SARS-CoV still has to be identified. Himalayan palm civets are suspected to be carriers of the virus, and these animals were associated epidemiologically with many, but not all, human SARS cases. In experiments, Himalayan palm civets were shown to be susceptible to SARS-CoV infection and replication. However, other animals like domestic cats and ferrets were also shown to be susceptible (Martina *et al.*, 2003; Wu *et al.*, 2005); and Chinese ferret badgers and racoon dogs were suggested as natural carriers of SARS-CoV-like viruses (Guan *et al.*, 2003). Our experiments demonstrated that only two amino acid residues of the receptor binding domain of SARS-CoV spike protein determine its efficiency to bind to its cell-surface receptor, ACE2. These two residues are variable in different animals and hence allow or prohibit SARS-CoV cell entry (Li *et al.*, 2005). This finding suggests that only few mutations need to occur in a naturally circulating SARS-CoV or SARS-CoV-like virus in the unknown reservoir to allow for cross-species transmission and further human SARS outbreaks. On the other hand, experiments performed in our and other laboratories also suggest that specific prevention and treatment of these SARS outbreaks could be achievable. In this review, we have presented strong support for the role of ACE2 as the sole cell-entry determinant of SARS-CoV, and we described the ability of neutralizing antibodies to disrupt the S-ACE2 interaction (Hattermann *et al.*, 2005; Li *et al.*, 2003b; Mossel *et al.*, 2005; Nie *et al.*, 2004; Sui *et al.*, 2004; Wang *et al.*, 2004). These results suggest that future vaccine candidates should elicit antibody responses to the S protein receptor binding domain. In terms of treatment, several promising research directions could be taken. First, based on our findings that the SARS-CoV S protein receptor binding domain can prevent cell transduction with S protein-pseudotyped lentiviruses (Wong *et al.*, 2004), modified polypeptides resembling S protein fragments could be developed to bind to ACE2 and prevent binding of SARS-CoV to its host cells. Second, the development of specific inhibitors of ACE2, which do not contain S protein fragments, is feasible. For example, the identification of an inhibitor, which prevents binding of SARS-CoV to ACE2 and ACE2 proteolytic activity, was recently

reported (Huentelman *et al.*, 2004). However, this inhibitor is most likely associated with severe side effects since it interrupts ACE2's physiological, as of yet unknown, function. We demonstrated that SARS-CoV binds to ACE2 at a site remote of the enzyme's active site (Li *et al.*, 2005), and we showed further that SARS-CoV binding does not interfere with ACE2's enzymatic activity (Li *et al.*, 2005; Moore *et al.*, 1997). Hence, better inhibitors could be identified, which only block S-ACE2 interaction but do not alter the physiological function of ACE2. Last, it has been shown that soluble ACE2 can prevent transduction of ACE2-expressing cells with SARS-CoV S protein-pseudotyped lentiviruses (Hofmann *et al.*, 2004; Li *et al.*, 2005), suggesting that a polypeptide consisting of the SARS-CoV-binding domain of ACE2 could be used as a therapeutic. We have shown that enzymatically inactive ACE2 is still able to interrupt S protein-mediated entry of lentiviral pseudotypes (Moore *et al.*, 2004). This should allow for the synthesis of ACE2 variants or fragments thereof, which would prevent SARS-CoV infection but would not interfere with the predicted role of ACE2 as a regulator of blood pressure (Yagil *et al.*, 2003).

The surprising discovery of ACE2 as a receptor of another coronavirus, HCoV-NL63, raises the question whether antivirals or vaccines could be developed, which target both this virus and SARS-CoV at the same time. Evidently, it is necessary to define the receptor-binding domain of HCoV-NL63's spike protein and the interacting region of ACE2 to find an answer.

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