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Angiotensin-converting enzyme 2: a functional receptor for SARS coronavirus

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Abstract. Cellular entry of enveloped viruses is often dependent on attachment proteins expressed on the host cell surface. Viral envelope proteins bind these receptors, and, in an incompletely understood process, facilitate fusion of the cellular and viral membranes so as to introduce the viral core into the cytoplasm. Only a small fraction of viral receptors have been identified so far. Recently, a novel coronavirus was identified as the etiological agent of severe acute respiratory syndrome (SARS). The fusion protein gene of SARS coronavirus (SARS-CoV) was cloned and characterized, and shortly thereafter, angiotensin-converting enzyme 2 (ACE2) was shown to be its functional receptor. Identification of ACE2 as a receptor for SARS-CoV will likely contribute to the development of antivirals and vaccines. It may also contribute to the development of additional animal models for studying SARS pathogenesis, and could help identify the animal reservoir of SARS-CoV.

Key words. Severe acute respiratory syndrome; SARS; coronavirus; angiotensin-converting enzyme 2; ACE2; viral fusion.

Introduction

The susceptibility of cells to viral infection is determined by their ability to support virus entry, replication, maturation and egress [1]. Virus entry depends on the expression of specific cellular receptors. In general, a range of proteins, carbohydrates and lipids can serve as virus receptors. The majority of enveloped viruses, i.e. viruses surrounded by a lipid bilayer derived from the host cell inner or outer membranes, achieve cellular entry by docking to specific membrane proteins that extend into the extracellular space. These highly diverse receptors usually react with viral fusion proteins called peplomers or spikes, which protrude from the viral surface. The viral core is released into the cytoplasm after fusion of the cellular membrane and the viral envelope [1]. Viral fusion proteins are usually expressed as precursor proteins into the endoplasmic reticulum, where they oligomerize and are cleaved by cellular proteases into receptor-binding and membrane fusion subunits, which remain bound to each other. Typically, they subsequently get transported to the cell surface or internal membrane systems [1].

Identification of viral receptors is crucial for understanding the host and tissue tropisms of a virus, which in turn shed light on the pathogenesis of viral diseases. Furthermore, variations in the receptor or its expression can contribute to differences in severity of disease. An understanding of viral entry might also allow development of substances that prevent viral binding to receptors or that freeze the entry process shortly thereafter. Despite considerable scientific interest, only a limited number of viral receptors have been identified so far [1].

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Coronaviruses

The viral family *Coronaviridae* is a member of the order *Nidovirales*, which additionally contains the *Arteriviridae* and *Roniviridae* [2, 3]. The viruses of these families share certain genomic features and transcription strategies but differ in morphology. Their most prominent shared feature is that they all biosynthesize a nested set of subgenomic messenger RNAs (mRNAs) – a strategy not seen in any other viral group [2]. Human pathogens are found only among viruses of the family *Coronaviridae*, which contains the two genera *Coronavirus* and *Torovirus* [2].

Coronaviruses are large, enveloped, spherical viruses about 100–120 nm in diameter, which contain single-stranded RNA genomes with positive polarity. The coronaviral RNA genome is bound to nucleocapsid (N) proteins. Together, they form a flexible, helical ribonucleocapsid, which is contained in an icosahedral inner core made of matrix (M) proteins. The lipid envelope, which surrounds the inner core, is formed during viral budding from intracellular membranes [4–6]. Coronaviruses received their name from their overall shapes, which, due to the large (20–40 nm) petal-shaped spikes protruding from the envelope, resemble crowns (Latin: *coronae*). With lengths between 27 and 32 kb, coronaviruses possess the largest genomes of all RNA viruses. The replication strategy of these viruses results in high-frequency recombination of their genomes. Coronaviruses infect a wide variety of species, including many mammals and birds. They cause both acute and chronic upper respiratory, gastrointestinal, hepatic and central nervous system (CNS) diseases [4–6].

Coronaviral spike proteins and receptors

The wide range of hosts infectable by coronaviruses is largely attributable to the variability of their spike proteins. Similarly, the virulence of coronavirus strains is also tightly associated with variations in these proteins [7–9]. Coronaviral spikes are type I membrane proteins which contain an N-terminal receptor-binding (S1) and a C-terminal membrane fusion (S2) domain [10]. The S2 domain contains amphipathic heptad repeats [10], which are predicted to engage in coiled-coil formation during cell-virus fusion. Based on models of cell fusion established with human immunodeficiency virus 1 (HIV-1) and influenza viruses, S1 binding to the receptor would induce exposure of a fusion peptide embedded in S2, which would then induce reorganization of the heptad repeats into coiled coils. Cellular membrane and viral envelope are thereby brought into close apposition [11–13]. Currently, two classes of fusion proteins are distinguished. Class I viral fusion proteins contain internal heptad repeat regions and an N-terminal fusion peptide. Class II proteins are heterodimers, lack these regions and possess internal fusion peptides [14]. Coronaviral spikes are similar to class I fusion proteins in that they are type I transmembrane proteins with heptad repeat regions synthesized in the endoplasmic reticulum and transported to the plasma membrane, but differ from class I fusion proteins in their lack of an N-terminal fusion peptide and the large size of their heptad repeat regions [15].

Three different genetic and serological groups (groups 1–3) of coronaviruses have been described. However, this classification is undergoing revision [16]. Until the discovery of SARS-CoV in 2003, only two coronaviruses were known to be pathogenic for humans. Human coronavirus 229E (HCoV-229E), a group 1 coronavirus, and HCoV-OC43, a group 2 coronavirus, usually cause mild upper respiratory infections ranging from self-resolving common colds to severe pneumonia in immunocompromised patients [17, 18]. The cellular receptor for HCoV-229E was identified as aminopeptidase N (APN, CD13) [19, 20]. Other group 1 coronaviruses that use this same zinc metalloprotease for cell entry include feline infectious peritonitis virus, porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus and canine coronavirus [21–24]. Recently, another human coronarvirus, designated HCoV-BL63, was isolated from an infant suffering from bronchiolitis and conjunctivitis [25, 26]. It remains to be seen whether this virus also utilizes APN as a cell-entry receptor. The receptor for HCoV-OC43 remains elusive. However, the receptor for another group 2 coronavirus, murine hepatitis virus (MHV), has been identified as a member of the pleiotropic family of carcinoembryonic antigen-cell adhesion molecules (CEACAMs), which are members of the immunoglobulin superfamily [27–30]. Bovine coronaviruses, which also belong to group 2, seem to bind to 9-*O*-acetylated sialic acids [31]. No receptors for group 3 coronaviruses, which typically have avian hosts, have been identified so far.

The SARS coronavirus and its receptor

In 2003, a novel coronavirus was identified as the etiological agent of severe acute respiratory syndrome (SARS), which had emerged in Southeast Asia [32–35]. SARS was recognized for the first time in 2002 when human inhabitants of Guangdong Province, China, presented with a flu-like disease characterized by pyrexia, myalgia, dyspnea and lymphopenia [36]. Many cases developed an acute pulmonary syndrome, which resulted in pneumonia, progressive respiratory failure and death in about 10% of cases. Spread of the virus occurred via airborne droplets and close contact with patients [37]. The natural reservoir of SARS-CoV remains elusive, but ferrets and domestic cats have been suggested as candidates because they are susceptible to infection and can transmit

the virus [38]. Chinese ferret badgers, raccoon dogs and Himalayan palm civets have also been suggested as reservoirs because they have been found infected with SARS-CoV-like viruses [39].

Initial analyses suggested that SARS-CoV does not belong to any of the three established coronaviral groups [40–43]. However, new data suggest that the virus clusters phylogenetically with group 2 coronaviruses [44, 45]. SARS-CoV possesses an RNA genome of about 30 kb. This genome begins with a putative leader sequence followed by two overlapping open reading frames, which encompass almost half the genome. A-1 ribosomal frameshift produces a polyprotein, which encodes nonstructural proteins like proteases, helicases and viral polymerase. The remainder of the genome encodes, among others, the viral structural components, i.e. the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins [32, 41, 42]. Analyses of the predicted amino acid sequence of SARS-CoV spike protein revealed a low (20–27%) pairwise amino acid identity to the spikes of other coronaviruses [42, 43], suggesting that this novel virus might use a different receptor than those identified for other coronaviruses.

Within months of publication of the SARS-CoV genome, a receptor, angiotensin-converting enzyme 2 (ACE2), was identified [46]. The approach taken was straightforward. ACE2 was immunoprecipitated from lysates of cells susceptible to SARS-CoV infection using the SARS-CoV S1 domain. The precipitated protein was then identified using mass spectrometry [46]. ACE2 has been demonstrated to bind SARS-CoV S1 specifically and with a high affinity of 1.7 nM [47]. Recombinant and cellularly expressed ACE2 supports the formation of large multinucleated cells with spike-protein-expressing cells, and ACE2 expression in cells that are usually not susceptible to SARS-CoV infection allows entry of live virus. Finally, antibodies to ACE2 block viral replication in Vero E6 cells [46].

ACE2 is a carboxy-metalloprotease that was independently discovered in 2000 by two groups. The enzyme is a type I transmembrane protein of 805 amino acids, which contains a single metalloprotease active site with an HEXXH zinc binding domain. It is only distantly related to APN, the receptor for group 1 coronaviruses [48, 49]. ACE2 transcripts were shown to be synthesized in human heart, kidney, testis [48], gastrointestinal tract and lungs [50]. Immunohistochemical examinations demonstrated high levels of ACE2 expression in the endothelium of intramyocardial and intrarenal vessels and in the renal tubular epithelium [48].

ACE2 appears to counter the physiological actions of the related ACE, which cleaves the inactive peptide angiotensin I to the highly potent vasoconstrictor angiotensin II. In fact, ACE2 has been shown to cleave angiotensin I to the metabolite angiotensin(1–9), which in turn is cleaved to angiotensin(1–7) [48, 51]. Angiotensin(1–7) has been identified as a vasodilator with antidiuretic effects. Recently, its receptor, the G-protein-coupled protooncogene Mas, has been identified [52]. ACE2 also cleaves des-arg-bradykinin, neurotensin and kinetensin [48]. The enzyme contains one catalytic domain that is 42% identical to each of the two respective domains of ACE. However, inhibitors of ACE like captopril or enalapril, which are used clinically to lower blood pressure, have no effect on the enzymatic activity of ACE2 [48, 49].

There is a considerable overlap of the tissue distribution of ACE2 with the distribution of SARS-CoV replication and the symptomatic manifestation of SARS [50]. For example, the primary site of SARS-CoV replication is the lungs [35], which have been shown to express ACE2 [46, 48, 50, 51]. ACE2 is highly expressed in gastrointestinal tract and kidneys, tissues from which SARS-CoV has been isolated [48, 50, 51]. However, ACE2 is strongly expressed in the human heart [48], but viral replication has not been detected in this organ. This discrepancy could be explained if SARS-CoV cell entry were dependent on cellular coreceptors, or other cellular factors, that are not well expressed in the heart. Alternatively, access of the virus to ACE2-expressing heart tissue may be limited.

Experiments imply that SARS-CoV cell fusion occurs at neutral pH and that no viral proteins other than the spike protein are required for the event [46, 54]. Curiously, the spike protein is not cleaved [46, 54], which is consistent with spike proteins of other group 1 coronaviruses [4] and in contrast to group 2 and 3 coronavirus spike proteins [15, 55, 56] and to the fusion proteins of other viruses. The ACE2 binding site has been localized to a small region in the S1 domain. A SARS-CoV S1 fragment consisting of only 193 amino acids (residues 318–510) binds ACE2 even more efficiently than the full-length SARS-CoV S1 domain [57, 58]. At least two amino acids (glutamic acid 452 and aspartic acid 454) of the S protein are crucial for ACE2 association. The receptor binding domain is distinct from those of MHV and HCoV-229E, which is consistent with the use of distinct receptors by these viruses [57].

An enzymatically inactive ACE2 variant still binds SARS-CoV spike protein and facilitates entry, implying that the proteolytic activity of ACE2 plays no significant role in SARS-CoV cell entry [46]. This is not surprising, since cell entry of the group 1 coronavirus TGEV is also independent of the proteolytic activity of its receptor, APN. In fact, neither inhibition of APN's catalytic site nor mutation of the site itself blocks TGEV cell entry [59]. It remains unclear why these different coronaviruses utilize diverse zinc metalloproteases as receptors.

Preliminary results also suggest that binding of SARS-CoV spike protein does not alter the enzymatic activity of ACE2 [J. H. Kuhn, unpublished]. It remains unclear whether spike protein binding leads to up- or downregu-

lation of ACE2 expression, to downstream signaling or to cleavage of any SARS coronaviral protein.

To simplify studies of SARS-CoV, a system using simian immunodeficiency virus (SIV) or murine leukemia virus (MLV) expressing green fluorescent protein (GFP) and pseudotyped with a codon-optimized form of SARS-CoV spike protein has been developed [60]. This system allows characterization of the interaction between ACE2 and SARS-CoV spike protein without using live SARS-CoV, obviating the requirement for stringent biosafety conditions. The system should facilitate the search for small-molecule inhibitors of receptor binding or entry and the testing of anti-sera to the SARS-CoV spike protein or its complex with ACE2. It may also be used to deliver target genes to ACE2-expressing cells.

Two therapeutic approaches have been shown effective in tissue culture. First, recombinant SARS-CoV spike protein or fragments thereof could be used as therapeutics, since they competitively inhibit binding of the virions to ACE2. The identification of a 193-amino-acid S1 fragment that binds to ACE2 [57] is a step in this direction. This fragment blocks cell entry of pseudotyped SIV at lower concentrations (50% inhibitory concentration (IC_{50}) of 10 nM under these experimental conditions) than the full-length S1 domain (IC_{50} of 50 nM). It should be noted, though, that these studies were performed using cells expressing unphysiologically high levels of ACE2 [57] and may underestimate the potency of this reagent in vivo. Efforts are under way to characterize the structure of this S1 fragment and to increase its affinity [S. W. Wong, unpublished]. Second, soluble recombinant ACE2 (sACE2) may also be used as a decoy to scavenge SARS-CoV in the bloodstream of infected patients [61]. ACE2 is most likely an essential regulator of blood pressure within local renin-angiotensin-aldosterone systems [52, 62], and also plays an important role in cardiovascular physiology [63, 64]. Thus, only catalytically inactive forms of sACE2 have potential as therapeutics for SARS, since only those forms would not interfere with regular physiological feedback systems. Such a mutated sACE2 can block infection of cells by an SIV pseudotype more potently than does the S1 fragment [60].

To date, there is no reliable animal model for SARS, although domestic cats, ferrets and monkeys have been proposed [32, 38]. Recently, murine ACE2 has been cloned and expressed. The molecule was determined to be 83% identical to its human counterpart [65]. Mice may be infectable with SARS-CoV, but their viral loads are low and they do not develop disease. Consistent with this observation, murine ACE2 binds to SARS-CoV S1 with low affinity [66]. However, robust SARS-CoV replication was observed after introducing human ACE2 into murine 3T3 cells, indicating that the cell entry step is the primary bottleneck of virus replication in mice [66]. This suggests that mice transgenic for human ACE2 may be a useful animal model. Similar studies with ACE2 of other animals may cast light on the animal reservoir of the virus.

Several questions remain outstanding. First, do zinc metalloprotease receptors in general offer some advantages to allow coronavirus cell entry, or is the finding of several viruses (HCoV-229E, SARS-CoV) using diverse variants of these enzymes (APN, ACE2) a coincidence? Second, does ACE2 cleave SARS-CoV proteins on the virion surface, perhaps generating bioactive peptides that contribute to SARS pathogenesis? Third, is ACE2 the only cellular factor permitting SARS-CoV cell entry or are coreceptors involved? Fourth, does the inflammatory response to SARS-CoV lead to upregulation of ACE2 expression in lung tissue? If previous progress in SARS research is any indication, answers to these questions will be forthcoming shortly.

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