Chapter 1
Cellular Entry of the SARS Coronavirus: Implications for Transmission, Pathogenicity and Antiviral Strategies

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Abstract A novel coronavirus was identified as the causative agent of the lung disease severe acute respiratory syndrome (SARS). The outbreak of SARS in 2002/2003 was associated with high morbidity and mortality and sparked international research efforts to develop antiviral strategies. Many of these efforts focussed on the viral surface protein spike (S), which facilitates the first indispensable step in the viral replication cycle, infectious entry into target cells. For infectious cellular entry to occur, the S protein must engage a cellular receptor, the carboxypeptidase angiotensin-converting enzyme 2 (ACE2). The interface between ACE2 and S protein, which has been characterized at the structural level, constitutes a key target for vaccines and inhibitors, and is believed to be an important determinant of viral pathogenesis and interspecies transmission. In this chapter, we will discuss how SARS-S mediates cellular entry and we will review the implications of this process for SARS coronavirus (SARS-CoV) transmission, disease development and antiviral intervention.

1.1 Introduction

The emergence of the severe acute respiratory syndrome coronavirus (SARS-CoV) in Guangdong Province, China, in 2002, and its subsequent spread in Asia and Canada clearly exemplified the vulnerability of societies and economies to a novel, highly pathogenic respiratory agent (Stadler et al. 2003; Peiris et al. 2003b). The outbreak, which was halted solely by the quarantine of exposed individuals and the use of conventional prevention measures such as surgical masks, was paralleled by an international, collaborative scientific effort to develop means for therapeutic and

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preventive intervention (Peiris et al. 2004; Stadler and Rappuoli 2005). The basis for the development of successful antiviral strategies is a thorough understanding of the molecular biology underlying viral amplification and pathogenesis, and many significant discoveries have been made in the SARS field since the identification of the virus early in 2003 (Drosten et al. 2003; Ksiazek et al. 2003; Peiris et al. 2003a). Several of these findings provided important insights into the structure and function of the viral spike (S) protein, which is used by the virus as the key to bind and enter host cells (Hofmann and Pöhlmann 2004). The most well-known examples are the identification of angiotensin-converting enzyme 2 (ACE2) as the host factor which is engaged by the viral S protein for infectious entry into cells, and the elucidation of the structure of the S protein receptor binding domain (RBD) in complex with ACE2 (Li et al. 2003, 2005a). These findings have major implications not only for vaccine and inhibitor development but also for our understanding of the SARS zoonosis, since adaptation of SARS-S to robust usage of human ACE2 was probably of key importance for efficient SARS-CoV spread in humans (Li et al. 2005a, 2005c). In this chapter, we will discuss how SARS-CoV gains access to target cells and how this process can be inhibited. In addition, we will review how the molecular interactions underlying SARS-CoV entry impact viral pathogenesis and interspecies transmission.

1.2 The Spike Protein: Key to the Host Cell

The SARS-Sprotein is a type I transmembrane protein, which comprises 1,255 amino acids and contains 23 consensus signals for *N*-linked glycosylation (Hofmann and Pöhlmann 2004). S protein is synthesized in the secretory pathway of infected cells. It contains an N-terminal signal sequence, which mediates import of the nascent protein into the endoplasmatic reticulum, where the protein is folded and modified with mannose-rich carbohydrates. Upon transport of the protein into the Golgi apparatus, most, if not all, of the high-mannose carbohydrates are processed into complex glycans (Nal et al. 2005). Evidence of *O*-glycosylation of SARS-S has not been reported. A novel dibasic ER retrieval motif in the cytoplasmic tail of SARS-S promotes accumulation of the S protein at the ER–Golgi intermediate compartment and the Golgi region (McBride et al. 2007), the sites where progeny particles are assembled (Stertz et al. 2007; Siu et al. 2008). Formation and budding of new particles are driven by the membrane protein (M), the envelope protein (E) and the nucleocapsid protein (N) (Huang et al. 2004; Hsieh et al. 2005; Siu et al. 2008); interactions with the M protein might facilitate S protein incorporation into particles. Trimers of the S protein protrude from the viral envelope and provide virions with a crown (Lat. *corona*) -like appearance, from which the name "coronaviruses" is derived.

The domain organization of SARS-S resembles that of several well-characterized viral membrane proteins, such as influenza virus hemagglutinin (HA) and human immunodeficiency virus (HIV) envelope protein (Env) (Hofmann and Pöhlmann 2004).

These proteins employ comparable strategies to facilitate fusion of viral and host cell membranes and are termed class I fusion proteins (Kielian and Rey 2006). They are distinguished from class II fusion proteins (Kielian 2006), found, for example, on flavi- and alphaviruses, by their distinct spatial organization and the particular configuration of the functional elements required for fusion with target cells: class I fusion proteins are inserted perpendicular to the viral membrane and contain an N-terminal surface unit (SU) and a C-terminal transmembrane unit (TM). The globular SU interacts with cellular receptors, while the TM promotes

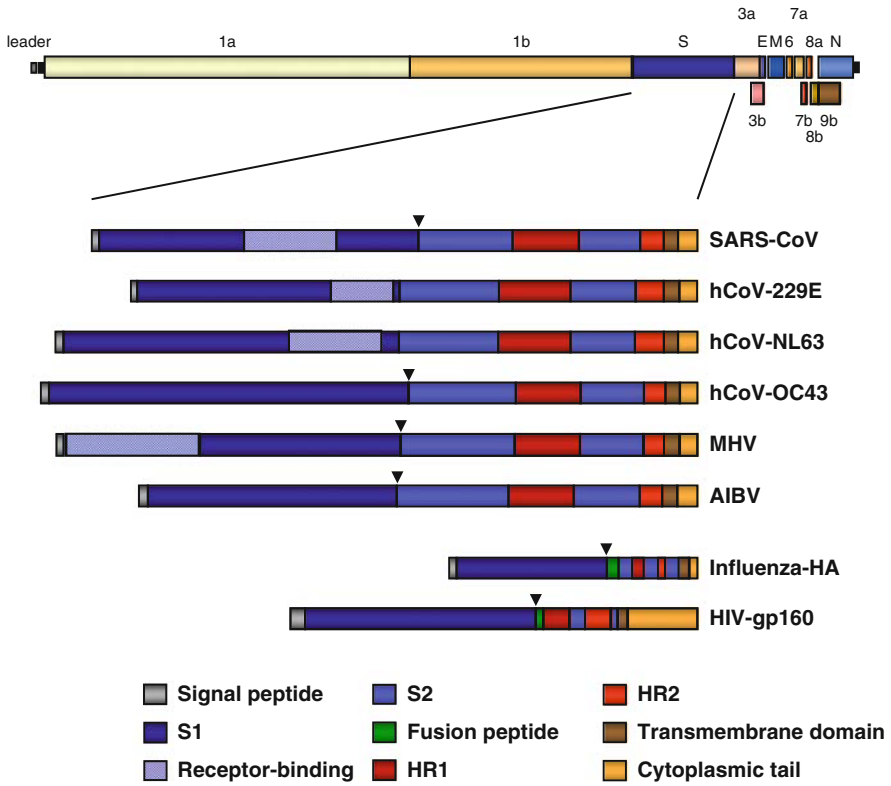

Fig. 1.1 Domain organization of coronavirus S proteins (adapted from Hofmann and Pöhlmann 2004). The position of the S protein open reading frame in the SARS-CoV genome is indicated in the *upper panel*. Coronavirus S proteins exhibit a domain organization characteristic for class I fusion proteins. The domain organization of prototype class I fusion proteins, the HIV envelope protein, and the influenza virus HA is shown below. A signal peptide is located at the N terminus and mediates import of the nascent protein into the secretory pathway of infected cells. The surface unit S1 contains a receptor binding domain (RBD), which allows engagement of cellular receptors for infectious entry. The transmembrane unit (S2) harbors functional elements pivotal to membrane fusion: a fusion peptide, two helical regions, and a transmembrane domain. Proteolytic cleavage into the S1 and S2 subunits by host-cell proteases is indicated by a *triangular arrow*. AIBV: avian infectious bronchitis virus; hCoV: human CoV; HR: helical region; MHV: murine hepatitis virus; SARS: severe acute respiratory syndrome

fusion of the viral and host cell membrane (Kielian and Rey 2006). The latter process depends on the presence of a fusion peptide and two helical regions (HR), conserved elements which are intimately involved in the membrane fusion process (Fig. 1.1), as discussed below. The S protein and the aforementioned fusion proteins are adapted to usage by different cellular receptors. Therefore, the SU (termed S1) of SARS-S does not exhibit appreciable sequence homology to the respective sequences of other class I fusion proteins. In contrast, the functional elements in TM, particularly the HRs, are conserved between different class I fusion proteins. Consequently, the TM (termed S2) of SARS-S shares homology with the corresponding sequences of other viral fusion proteins (Hofmann and Pöhlmann 2004), which has important implications for development of antiviral strategies, as discussed below.

1.3 The Attachment Factors DC-SIGN and DC-SIGNR: Enhancers or Inhibitors of SARS-CoV Infection?

The interaction of SARS-S with ACE2 is the first indispensable step in the entry cascade, as discussed below. It needs to be noted, however, that SARS-S also binds to other cell surface factors and these interactions, although being ultimately dispensable for infectious entry, might profoundly alter infection efficiency. Thus, it is well established that the calcium-dependent (C-type) lectin dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN, CD209; Geijtenbeek et al. 2000) and the related protein DC-SIGNR (L-SIGN, CD209L; Pöhlmann et al. 2001; Bashirova et al. 2001) bind to SARS-S (Marzi et al. 2004; Yang et al. 2004; Jeffers et al. 2004; Khoo et al. 2008) in a glycan-dependent fashion (Shih et al. 2006; Han et al. 2007). DC-SIGNR was detected in the lung on ACE2-positive, SARS-CoV-infected cells and on uninfected bystander cells (Chan et al. 2006), while DC-SIGN expression was found to be induced upon SARS-CoV infection (Yen et al. 2006), suggesting that these lectins might have ample opportunity to capture SARS-CoV in infected individuals.

Despite the potentially important role of DC-SIGN/R in SARS-CoV infection, the consequences of DC-SIGN/DC-SIGNR (collectively referred to as DC-SIGN/R) engagement by SARS-S for viral infectivity are largely unclear. One group suggested that DC-SIGN/R function as bona fide receptors which facilitate viral entry into otherwise nonpermissive HeLa cells (Han et al. 2007). DC-SIGNR was also identified in a functional screen for receptors used by SARS-CoV for cellular entry (Jeffers et al. 2004), further suggesting that DC-SIGNR might support viral entry, at least under certain conditions and probably with low efficiency. In contrast, three other studies failed to detect an appreciable receptor function for DC-SIGN/R but suggested that these proteins might enhance infectious entry into ACE2-expressing cell lines (Marzi et al. 2004; Yang et al. 2004; Shih et al. 2006). Finally, and in contrast to all aforementioned studies, Chan and co-workers

provided evidence that DC-SIGNR plays a protective role in SARS-CoV infection (Chan et al. 2006). Thus, it was demonstrated that DC-SIGNR-dependent uptake of SARS-CoV into cell lines might lead to viral degradation and might thus reduce viral infectivity for target cells (Chan et al. 2006). In agreement with this finding, evidence was obtained that the combination of certain DC-SIGNR allelic variants, which resulted in reduced SARS-CoV uptake in cell culture, was associated with increased risk of SARS-CoV infection in humans (Chan et al. 2006), albeit these findings are not undisputed (Tang et al. 2007; Zhi et al. 2007). In any case, most functional studies described above have in common that they were carried out with cell lines, which do not adequately model type II pneumocytes, the major targets of SARS-CoV infection (Hamming et al. 2004; Ding et al. 2004; To and Lo 2004; Mossel et al. 2008), and further work with primary lung epithelium is required to help to elucidate the role of DC-SIGN/R in SARS-CoV infection. Notably, a single study examined the impact of DC-SIGN/R-specific antibodies on viral spread in primary human airway epithelium cultured at the air–liquid interface and observed no inhibition (Sims et al. 2008), although it was not investigated if these lectins were indeed expressed by the cells examined. Finally, it is worth mentioning that SARS-S binds to lectins other than DC-SIGN/R, such as the C-type lectin LSECtin (Gramberg et al. 2005) which is largely co-expressed with DC-SIGNR, and the consequences of these interactions for viral amplification have not been determined. Collectively, it is clear that binding to DC-SIGN/R and related lectins has the potential to modulate viral spread in vivo. It remains to be determined, however, if lectin binding augments or suppresses viral replication. Recently described knock-in mice for human DC-SIGN (Schaefer et al. 2008) or SIGNR1 (a murine homologue of human DC-SIGN) knock-out mice (Lanoue et al. 2004) might be useful to clarify these questions.

1.4 The Two Faces of ACE2: SARS-CoV Receptor and Protector Against Lung Damage

In contrast to attachment factors, cellular receptors are indispensable for infectious viral entry. In order to discover such factors, several laboratories used the soluble SARS-S1 subunit for co-immunoprecipitation of cellular binding partners. A milestone study by Li and colleagues identified the carboxypeptidase ACE2, an integral part of the renin–angiotensin system (see below), as a high-affinity SARS-S interactor (Li et al. 2003). Ectopic expression of ACE2 on barely permissive 293T cells facilitated efficient SARS-S-dependent cell–cell and virus–cell fusion (Li et al. 2003), suggesting that ACE2 might play an important role in SARS-CoV entry. Similar results were obtained by an independent study (Wang et al. 2004), which used a comparable approach to identify cellular binding partners of SARS-S. Subsequently, it was shown that endogenous expression of ACE2 correlates with susceptibility to SARS-CoV infection of cell lines (Nie et al. 2004; Hofmann et al.

2004a) and that ectopic expression of ACE2 facilitates SARS-S-driven infection of otherwise nonsusceptible cells (Mossel et al. 2005). Moreover, it was demonstrated that SARS-CoV infects ACE2-positive type II pneumocytes and ACE2-positive cells in the intestinal epithelium (Hamming et al. 2004; Ding et al. 2004; To and Lo 2004; Chan et al. 2006; Mossel et al. 2008), albeit ACE2-independent infection of target cells has also been suggested (Gu et al. 2005; Gu and Korteweg 2007). Finally, knock-out of ACE2 in mice was found to largely abrogate susceptibility to SARS-CoV infection (Kuba et al. 2005), indicating that ACE2 functions as a bona fide SARS-CoV receptor, which is necessary and sufficient for infectious entry into target cells.

1.4.1 The Structure of the Interface Between SARS-S and ACE2

A thorough understanding of the interface between SARS-S and ACE2 is key to the development of antiviral strategies targeting viral entry. The domains and amino acid residues in SARS-S and ACE2, which contribute to the efficient interaction of these proteins, were initially mapped by mutagenic analyses. These studies identified amino acids 318–510 in SARS-S as an independently folded RBD, which binds to ACE2 with higher affinity than the full length S protein (Xiao et al. 2003; Wong et al. 2004; Babcock et al. 2004). The RBD was also shown to be the major target of neutralizing antibodies (He et al. 2004a, 2004b, 2005), and several residues within amino acids 450–490 were suggested to be critical for optimal ACE2 engagement (Wong et al. 2004; Li et al. 2005c). In addition, exploitation of species-specific differences in murine, rat and human ACE2 allowed the mapping of certain amino acid residues, particularly L353, as important for receptor function (Li et al. 2004, 2005c). These results were supported and extended by the subsequent solution of the structure of the RBD in complex with ACE2 (Li et al. 2005a): the RBD consists of a core (a five-stranded antiparallel β-sheet), and an extended loop, which contains all amino acids making contacts with ACE2. The extended loop, also termed receptor-binding motif (RBM), comprises amino acids 424–494 (Li et al. 2005a), and thus includes the residues defined by mutagenic analysis to be important for SARS-S interactions with ACE2 (Wong et al. 2004; Li et al. 2005c). The RBM contacts the N-terminal helix of ACE2 and the loop between helices α2 and α3. Moreover, a portion of the RBM inserts between a short helix in ACE2 (amino acids 329–333) and a β-hairpin at ACE2 residue L353, supporting the previously postulated contribution of L353 to appropriate spike–receptor interactions (Li et al. 2005c). Conformational changes inherent to the peptidase activity of ACE2 do not impact the availability of the S protein binding site (Li et al. 2005a), in agreement with the observation that an ACE2 inhibitor which blocks peptidase activity and arrests ACE2 in a closed conformation does not inhibit SARS-S-dependent entry (Towler et al. 2004; Li et al. 2005c). Collectively, the functional and structural studies defined amino acids in SARS-S and ACE2, which facilitate the tight association of these proteins. In addition, the results highlighted that natural variation

of these sequences might have important implications for SARS-CoV transmission and pathogenicity, as discussed below.

1.4.2 Sequence Variations at the SARS-S/ACE2 Interface Might Impact Viral Transmission and Pathogenicity

Horseshoe bats harbor SARS-CoV-related viruses and might constitute the natural reservoir of SARS-CoV (Lau et al. 2005; Li et al. 2005b). However, the sequence homology between bat and human viruses is limited (Lau et al. 2005; Li et al. 2005b). Thus, the S protein of animal viruses does not contain an RBM-like sequence and does not use ACE2 for cellular entry (Ren et al. 2008). It is therefore probable that SARS-CoV was introduced into the human population via an intermediate host, and palm civets, which harbor viruses with high sequence homology to human SARS-CoV, are possible candidates (Guan et al. 2003; Song et al. 2005). Notably, the S proteins of human viruses from the 2002/2003 epidemic bind human ACE2 with much higher efficiency than their palm civet counterparts (Li et al. 2005c), indicating that efficient spread in humans required adaptation of the SARS-S sequence. Indeed, sequence comparison revealed that the civet RBD contains four amino acid changes relative to the human sequence. Two of these changes are located outside the RBM and do not impact receptor interactions (Li et al. 2005c). In contrast, the remaining two changes, N (human) to K (palm civet) at position 479 and T (human) to S (palm civet) at position 487, afflicted residues making direct contact with ACE2 and significantly decreased binding to human ACE2 (Li et al. 2005a, 2005c). Thus, N479 and T487 might be required for efficient spread in and between humans (Li et al. 2005a, 2005c; Li 2008). Interestingly, viruses isolated from sporadic SARS cases in the winter of 2003/2004, which were not associated with severe disease or human-to-human transmission, contained a serine at position 487 (Li et al. 2005a), further indicating that this amino acid might play a key role in human-to-human transmission and viral pathogenicity.

The potential for zoonotic transmission of SARS-CoV might also be determined by species-specific variations in the ACE2 sequence. Thus, murine and rat ACE2, which do not (rat), or only inefficiently (murine), support SARS-S-driven entry (Li et al. 2004, 2005c), contain a leucine (human) to histidine (mouse, rat) exchange at position 353. This exchange impedes formation of robust contacts with T487 in SARS-S and thereby prevents murine and rat ACE2 from efficiently supporting SARS-S-driven cellular entry (Li et al. 2005a). In addition, the rat but not the murine receptor contains a M82N exchange, which introduces a glycosylation signal. The glycan added to N82 blocks the interaction with L472 in SARS-S and further decreases receptor function, explaining why rat ACE2 is less capable of supporting SARS-S-driven entry than murine ACE2 (Li et al. 2005a). These results, in conjunction with the aforementioned variations in the RBD sequence, highlight that the efficiency of the SARS-S interaction with ACE2 might be a critical determinant of interspecies transmission of SARS-CoV.

1.4.3 The Human Coronavirus NL63 Uses ACE2 for Cellular Entry

A novel human coronavirus, NL63, was discovered by two Dutch groups in the aftermath of the SARS-CoV outbreak (van der Hoek et al. 2004; Fouchier et al. 2004). NL63 is a group I CoV and shows high sequence similarity to the long-known human CoV 229E. The 229E virus, like all other group I viruses described at the time of the NL63 discovery, uses CD13 (aminopeptidase N) as a receptor for cellular entry (Hofmann and Pöhlmann 2004). Considering the specificity of group I viruses for CD13 and taking into account that the spike proteins of 229E and NL63 share 56% sequence identity (van der Hoek et al. 2004; Pyrc et al. 2004), it was surprising that NL63 was shown to use ACE2 and not CD13 for cellular entry (Hofmann et al. 2005). This finding raised the question of whether both viruses use similar strategies to engage ACE2. Mapping studies revealed that an N-terminal unique region in NL63-S, which was suspected to function as RBD, is in fact dispensable for receptor engagement (Hofmann et al. 2006). In contrast, several motifs within amino acids 232 and 684 were found to be required for ACE2 binding within an initial study, and it was suggested that NL63-S might not harbor a single continuous RBD (Hofmann et al. 2006). However, subsequent analyses narrowed the region responsible for ACE2 binding to amino acids 301–643 and 476–616, respectively, and a SARS-S RBM-like motif was identified in NL63-S (Li et al. 2007; Lin et al. 2008). Several amino acid substitutions in ACE2 were found to alter ACE2 usage by SARS-S but not by NL63-S (Hofmann et al. 2006), indicating that both S proteins might interact with different ACE2 surfaces. This interpretation is not undisputed (Li et al. 2007) and solution of the structure of NL63-S in complex with ACE2 might be required to clarify whether SARS-S and NL63-S recognize ACE2 differentially. In any case, it is clear that both viruses employ different mechanisms to activate membrane fusion once the S proteins have bound to ACE2. Thus, it is believed that upon ACE2 engagement SARS-CoV is internalized into endosomal vesicles, where the pH-dependent cellular protease cathepsin L activates SARS-S by cleavage (Simmons et al. 2005). In contrast, low pH and cathepsin activity seem to be largely dispensable for NL63-S-driven entry and it is at present unclear how NL63-S-driven membrane fusion is triggered (Huang et al. 2006; Hofmann et al. 2006).

1.4.4 SARS Versus NL63: A Correlation Between ACE2 Downregulation and Viral Pathogenicity?

NL63 is a globally distributed pathogen which is acquired early in childhood and does not usually cause severe disease (Pyrc et al. 2007). This observation contrasts with the high pathogenicity of SARS-CoV and raises the question of which viral factors determine disease severity. Again, S protein interactions with ACE2 might

play a central role. Thus, a milestone discovery by Imai and colleagues indicated that ACE2 expression protects against development of acute respiratory distress syndrome (ARDS) (Imai et al. 2005). ACE2 is an integral component of the renin–angiotensin system (RAS), a key regulator of blood pressure and, as demonstrated by Imai and colleagues (Imai et al. 2005), lung function (Imai et al. 2008; Penninger et al. 2008). Knock-down of ACE2 in a mouse model caused accumulation of angiotensin II, which promoted development of ARDS by signaling via the AT1R receptor (Imai et al. 2005, 2008; Penninger et al. 2008). Conversely, inhibition of AT1R and application of soluble ACE2 protected against ARDS (Imai et al. 2005). Interestingly, a soluble form of the S1 subunit of SARS-S was shown to down-regulate ACE2 expression in vitro and in vivo (Kuba et al. 2005), indicating that SARS-S engagement of ACE2 might promote SARS development even in the absence of productive infection. The S protein of NL63 exhibits a markedly reduced affinity for ACE2 compared to SARS-S (Mathewson et al. 2008) and seems to engage the receptor in a different fashion (Hofmann et al. 2006), suggesting that differential ACE2 downregulation by SARS-CoV and NL63 could contribute to the differential pathogenicity of these viruses. However, it is largely unclear how SARS-CoV decreases ACE2 expression and the effect of NL63 on ACE2 levels has not been systematically investigated. Notably, a recent study indicates that SARS-S might promote shedding of the ACE2 ectodomain by inducing ACE2 cleavage by TACE/ADAM17 (Fig. 1.2), a process that seems to be essential for

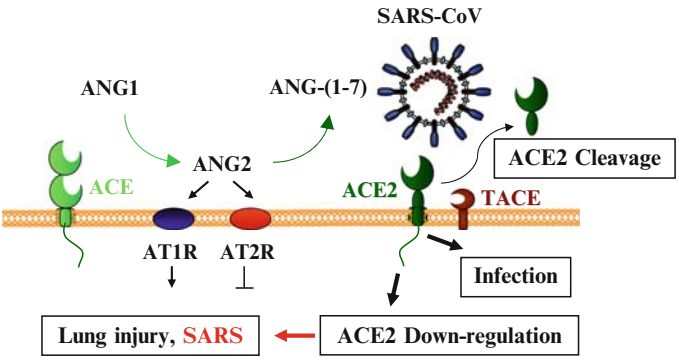

Fig. 1.2 ACE2 downregulation by SARS-S might promote development of SARS (adapted from Kuba et al. 2006). ACE and ACE2 are key components of the renin–angiotensin system. ACE processes angiotensin I (ANG1) into angiotensin II (ANG2) and accumulation of ANG2 can promote acute lung failure via angiotensin II type 1 receptor (AT1R). This process is prevented by ACE2, which converts ANG2 into angiotensin 1-7 (ANG-(1-7)). The angiotensin II type 2 receptor (AT2R) also exerts a protective function. The interactions of SARS-S with ACE2 drive infectious entry but also induce downregulation of ACE2, possibly by promoting ACE2 cleavage by TACE/ADAM17. Diminished ACE2 expression then facilitates SARS development. ACE: angiotensin-converting enzyme; TACE: TNF-α converting enzyme; ADAM17: ADAM metallopeptidase domain 17

infectious entry (Haga et al. 2008). In contrast, NL63-S did not induce appreciable ACE2 shedding (Haga et al. 2008). Thus, the previously observed ACE2 down-regulation by SARS-S might have been due to proteolytic cleavage and dissociation of the ectodomain rather than ACE2 internalization and degradation. However, it is unclear if shedding of the ACE2 ectodomain actually exacerbates SARS development, considering that soluble ACE2 protects against ARDS in a mouse model (Imai et al. 2005).

1.5 Cleavage by Endosomal Cathepsin Proteases Activates SARS-S

Class I fusion proteins usually require proteolytic cleavage to transit into an activated state (Hofmann and Pöhlmann 2004; Kielian and Rey 2006). However, the strategies to accomplish proteolytic activation can vary. Many fusion proteins are cleaved by subtilisin-like proteases in the secretory pathway of infected cells, and proteolytically processed proteins are incorporated into virions. This applies to the S proteins of most strains of murine hepatitis virus (MHV), a group II coronavirus. The membrane fusion reaction is subsequently triggered by binding of the cleaved S proteins to their cellular receptor, CEACAM-1 (Williams et al. 1991; Nash and Buchmeier 1997; de Haan et al. 2004; Qiu et al. 2006). Consequently, entry is pH-independent and encompasses fusion of the viral membrane with the plasma membrane of target cells (Nash and Buchmeier 1997; de Haan et al. 2004; Qiu et al. 2006). The influenza virus HA is either cleaved by subtilisin proteases in the secretory pathway or by secreted proteases present in the lung lumen. However, subsequent binding to the receptor determinant sialic acid does not trigger membrane fusion but internalization into endosomal vesicles, where fusion is triggered by low pH (Eckert and Kim 2001). Thus, infectious entry of influenza viruses is pH-dependent and is facilitated by fusion of the viral membrane with endosomal membranes (Eckert and Kim 2001).

The SARS-S protein employs a mixture of the entry strategies described above. At present, there is no evidence for appreciable cleavage of SARS-S produced in infected cells (Xiao et al. 2003; Yang et al. 2004; Simmons et al. 2004; Yao et al. 2004; Hofmann et al. 2004b), with the exception of a single report (Wu et al. 2004). It has been documented that the presence of furin can augment SARS-S activity and that a furin inhibitor blocks SARS-CoV infection (Bergeron et al. 2005; Follis et al. 2006). However, cleavage of SARS-S has not been detected under these conditions (Bergeron et al. 2005; Follis et al. 2006). Instead, a seminal study by Simmons and colleagues showed that SARS-S is activated by the endosomal, pH-dependent protease cathepsin L upon uptake into target cells, and that cathepsin L activity is essential for infectious entry (Simmons et al. 2005). Cathepsin B can also contribute to SARS-S activation but seems to be of minor importance compared to cathepsin L (Simmons et al. 2005). Importantly, appropriate SARS-S cleavage by cathepsin L seems to require a modest conformational rearrangement of SARS-S (Simmons

et al. 2005), which is induced upon binding to ACE2 (Beniac et al. 2007). Thus, SARS-S-driven entry is pH-dependent and relies on fusion of viral and endosomal membranes (Yang et al. 2004; Simmons et al. 2004, 2005; Hofmann et al. 2004b). However, acidic conditions are required for cathepsin activity and have no triggering effect on SARS-S (Simmons et al. 2005).

The cathepsin L cleavage site in SARS-S was mapped to T678, when recombinant proteins were employed (Bosch et al. 2008), but evidence that T678 is important for SARS-CoV entry is lacking and cathepsin L-mediated cleavage of virion-associated SARS-S in target cells remains to be demonstrated. It is also unclear if cellular proteases other than cathepsin B and L can allow SARS-S-driven entry into certain target cells. An activating function of factor Xa has recently been suggested (Du et al. 2007) but the results await confirmation. Finally, it is noteworthy that engineered cleavage of SARS-S in virus-producing cells can ablate the need for cathepsin activity in target cells (Watanabe et al. 2008). This finding highlights the need to analyze if SARS-S is cleaved in primary lung cells and to determine if cathepsin activity is indeed required for viral spread in vivo – information pivotal to efforts aiming at the development of cathepsin inhibitors for antiviral therapy.

1.6 Membrane Fusion is Driven by Conserved Elements Located in the S2 Subunit of the SARS Spike Protein

The functional organization of SARS-S2 resembles that of the TMs of other class I fusion proteins and SARS-S-driven membrane fusion reaction follows the principles previously established for other class I fusion proteins (Hofmann and Pöhlmann 2004): membrane fusion commences by insertion of the fusion peptide into the target cell membrane (Fig. 1.3). In this context, it is worth noting that SARS-S, in contrast to, for example, HIV Env and influenza HA, contains an "internal" fusion peptide, which does not constitute the N terminus of S2 but may comprise amino acids 770–788 (Sainz et al. 2005). Upon fusion peptide insertion, the S2 subunit is connected with the viral and the target cell membrane. Subsequently, the C-terminal HR (termed HR2) folds back onto the N-terminal HR (termed HR1), forming an energetically stable six-helix bundle structure, in which HR1 and HR2 are oriented in an antiparallel fashion (Bosch et al. 2003; Tripet et al. 2004; Liu et al. 2004; Supekar et al. 2004; Ingallinella et al. 2004; Xu et al. 2004; Hsu et al. 2004). Thereby, viral and target cell membranes are pulled into close proximity, allowing the membranes to merge (Fig. 1.3). Peptides derived from HR2, which bind to HR1 and block the formation of the six-helix bundle, are used for therapy of HIV infection (Este and Telenti 2007). A similar approach was successful for blockade of SARS-CoV spread in cell culture (Bosch et al. 2003; Liu et al. 2004; Zhu et al. 2004; Yuan et al. 2004; Ni et al. 2005), but the inhibitors developed were not as potent as those used to treat HIV infection. One reason

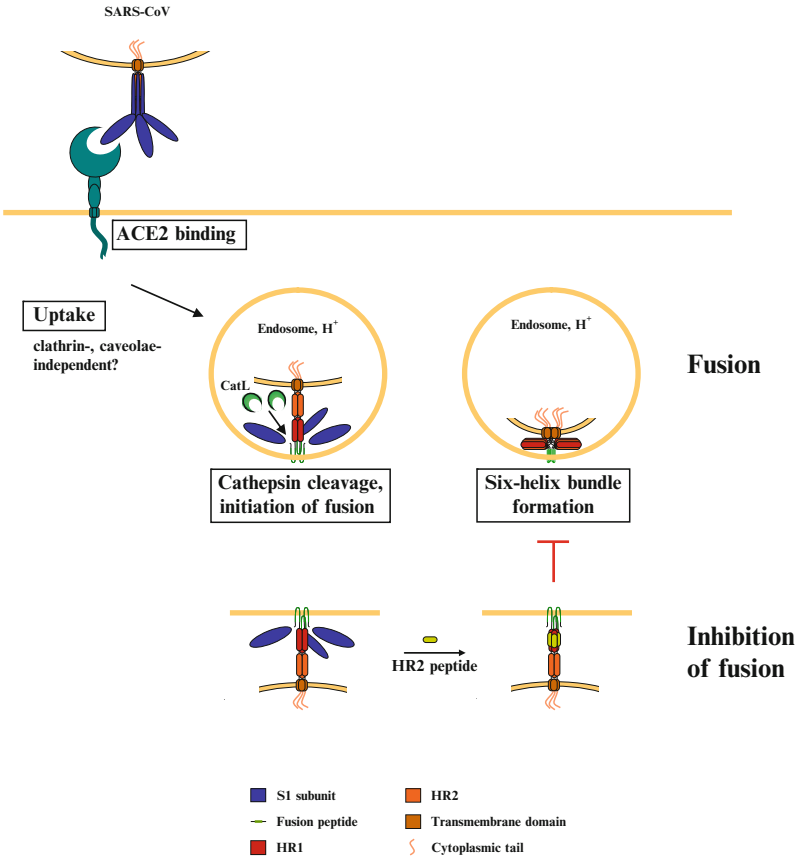

Fig. 1.3 Cellular entry of SARS-CoV and its inhibition (adapted from Hofmann and Pöhlmann 2004). The cellular entry of SARS-CoV commences by binding of the S protein to its receptor ACE2. Bound virus is then taken up into target cells, possibly by a clathrin- and caveolae-independent mechanism (Wang et al. 2008). The S protein is cleaved by the pH-dependent cellular protease cathepsin L in endosomes, and cathepsin L activity is essential for infectious entry. The membrane fusion reaction starts with the insertion of the fusion peptide into the target cell membrane. Formation of the stable six-helix bundle structure brings the viral and the target cell membrane into close proximity and is intimately associated with membrane fusion. The fusion reaction can be inhibited by HR2-derived peptides, which bind into a groove on HR1 and thereby prevent back-folding of HR2 onto HR1 and thus the formation of the six-helix bundle structure

for the decreased potency might be inherent to the cellular location of the membrane fusion reaction: the HIV Env protein drives fusion with the plasma cell membrane, and the target of the inhibitory peptides is readily accessible. In contrast, SARS-CoV fuses with endosomal membranes, and inhibitors must be taken up into endosomes to efficiently block the fusion reaction. Potentially, this could present a significant hurdle to the development of fusion inhibitors for therapy of SARS-CoV infection (Watanabe et al. 2008).

1.7 Conclusions

The cellular entry of SARS-CoV is a multistep process which involves the formation of several transient intermediates. All structures participating in the entry cascade are potential targets for inhibitors and the feasibility of several approaches to prevent entry has already been demonstrated. The first step, SARS-S engagement of ACE2, is an attractive target for both preventive and therapeutic approaches. Thus, the immunization with the RBD has been shown to elicit neutralizing antibodies (He et al. 2004a, 2005), and monoclonal RBD-specific antibodies which exhibit potent antiviral effects in animal models have been identified (Sui et al. 2004, 2005; Rockx et al. 2007; Zhu et al. 2007). In addition, SARS-S binding to ACE2 can be inhibited by nonpeptidic molecules targeting the receptor (Huentelman et al. 2004), and soluble ACE2 was shown to block SARS-CoV infection (Hofmann et al. 2004a) and to protect against ARDS (Kuba et al. 2005), making this approach particularly promising. The S protein can also be targeted by lectins, which bind glycans on the S protein and thereby block viral entry (van der Meer et al. 2007; Keyaerts et al. 2007), albeit issues concerning potential toxicity and antigenicity remain to be addressed. After binding to ACE2 and uptake into target cells, the S protein must be activated by cathepsin L, and potent cathepsin L inhibitors are available (Simmons et al. 2005). However, the role of cathepsin L in viral spread in vivo remains to be assessed, and knock-out mice (Reinheckel et al. 2001) might be suitable tools for these endeavours. Finally, inhibitors of the fusion reaction have been described (Bosch et al. 2003; Liu et al. 2004; Zhu et al. 2004; Yuan et al. 2004; Ni et al. 2005), but optimization of available compounds and generation of nonpeptidic compounds is desirable. In summary, the approaches described above, particularly combinations thereof, should allow development of compounds suitable for effectively preventing or combating future outbreaks of SARS-CoV.

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