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Cellular entry of the SARS coronavirus

Heike Hofmann and Stefan Pöhlmann

Institute for Clinical and Molecular Virology and Nikolaus-Fiebiger-Center, University of Erlangen-Nürnberg, 91054 Erlangen, Germany

Enveloped viruses have evolved membrane glycoproteins (GPs) that mediate entry into host cells. These proteins are important targets for antiviral therapies and vaccines. Several efforts to understand and combat infection by severe acute respiratory syndrome coronavirus (SARS-CoV) have therefore focused on the viral GP, known as spike (S). In a short period of time, important aspects of SARS-CoV S-protein function were unraveled. The identification of angiotensin-converting enzyme 2 (ACE2) as a receptor for SARS-CoV provided an insight into viral tropism and pathogenesis, whereas mapping of functional domains in the S-protein enabled inhibitors to be generated. Vaccines designed on the basis of SARS-CoV S-protein were shown to be effective in animals and consequently are attractive candidates for vaccine trials in humans. Here, we discuss how SARS-CoV S facilitates viral entry into target cells and illustrate current approaches that are used to inhibit this process.

In November 2002, a new form of infectious pneumonia, known as severe acute respiratory syndrome (SARS), emerged in Guangdong province, mainland China. Aided by a delayed response from public health officials, the disease experienced a global spread in 2003. Patients were diagnosed with SARS in 29 countries, with the majority of cases being reported in Asia. In the absence of effective treatment, the disease was combated solely using quarantine measures and travel restrictions. However, these strategies proved effective and the SARS epidemic was stopped in June 2003. By then, the death toll of SARS had accumulated to 774 out of 8098 infected patients and the disease had resulted in enormous economic damage [1].

The attempts to contain SARS were paralleled by a multi-centered, collaborative effort by scientists to identify and analyze the infectious agent behind the disease. These investigations culminated in the identification of a novel coronavirus (CoV) in SARS patients [2–4], which was followed by the demonstration that this virus was the cause of the disease [5,6]. When the sequence of the new virus became publicly available shortly after the discovery of SARS-CoV [7,8], the groundwork had been laid to analyze and combat SARS on a molecular level.

The virus has not reemerged since July 2003, except for several cases of laboratory-acquired infections and one natural outbreak resulting in four infected people, suggesting that SARS will probably not develop into a seasonal disease. However, SARS-CoV-related viruses have been detected in game animals that are commonly sold in Chinese markets [9], and it is probable that the virus replicates in an animal reservoir. Therefore, SARS-CoV could be reintroduced into the human population at any time. To be prepared for such a scenario, the development of vaccines and therapeutics is an important task.

Infection of host cells by SARS-CoV is initiated by the interaction of the viral spike (S) protein with receptors on the cell surface; this is an essential step for viral replication. Because of its crucial role in CoV spread, the S-protein is a major focus of SARS research. In this review, we will describe how S interacts with cellular factors to drive viral entry and how this process can be inhibited.

The S-protein drives entry into target cells

CoVs are subdivided into three phylogenetic groups; human CoVs are found in groups I (229E and NL63) and II (OC43). Depending on the method of sequence analysis used, SARS-CoV either constitutes a new phylogenetic group or a subgroup of group II CoVs [10,11]. The organization of the positive-stranded RNA genome of SARS-CoV, which is 29.7 kb in size, resembles that of other coronaviruses and harbors open-reading frames (ORFs) that encode a large polyprotein required for genome replication, four structural proteins and eight additional polypeptides of unclear function (Figure 1) [1]. Replication of SARS-CoV is briefly described in Box 1. The S, M and E proteins are incorporated into the viral envelope, and S-protein dimers or trimers protrude from the viral membrane, providing CoVs with a characteristic corona-resembling shape.

Infection of target cells by CoVs is driven by the S-protein, which facilitates attachment to target cells and promotes fusion of the viral and the cellular membranes, thereby allowing the insertion of the viral genome into the cellular cytoplasm. The CoV S-proteins are organized into an outer subunit (known as S1), which harbors the receptor-binding domain, and a transmembrane subunit (known as S2), which contains functional elements involved in membrane fusion (Figure 1). Notably, glycoproteins (GPs) of highly divergent viruses, including human immunodeficiency virus (HIV), exhibit a comparable architecture (Figure 1). These GPs, referred to as class I fusion proteins, use similar mechanisms to promote membrane fusion, which has important implications for therapeutic intervention.

Despite the similarities in domain organization, the SARS-CoV S-protein does not exhibit significant sequence identity with any other CoV S-protein; the highest

Corresponding author: Stefan Pöhlmann (stefan.poehlmann@viro.med.uni-erlangen.de).

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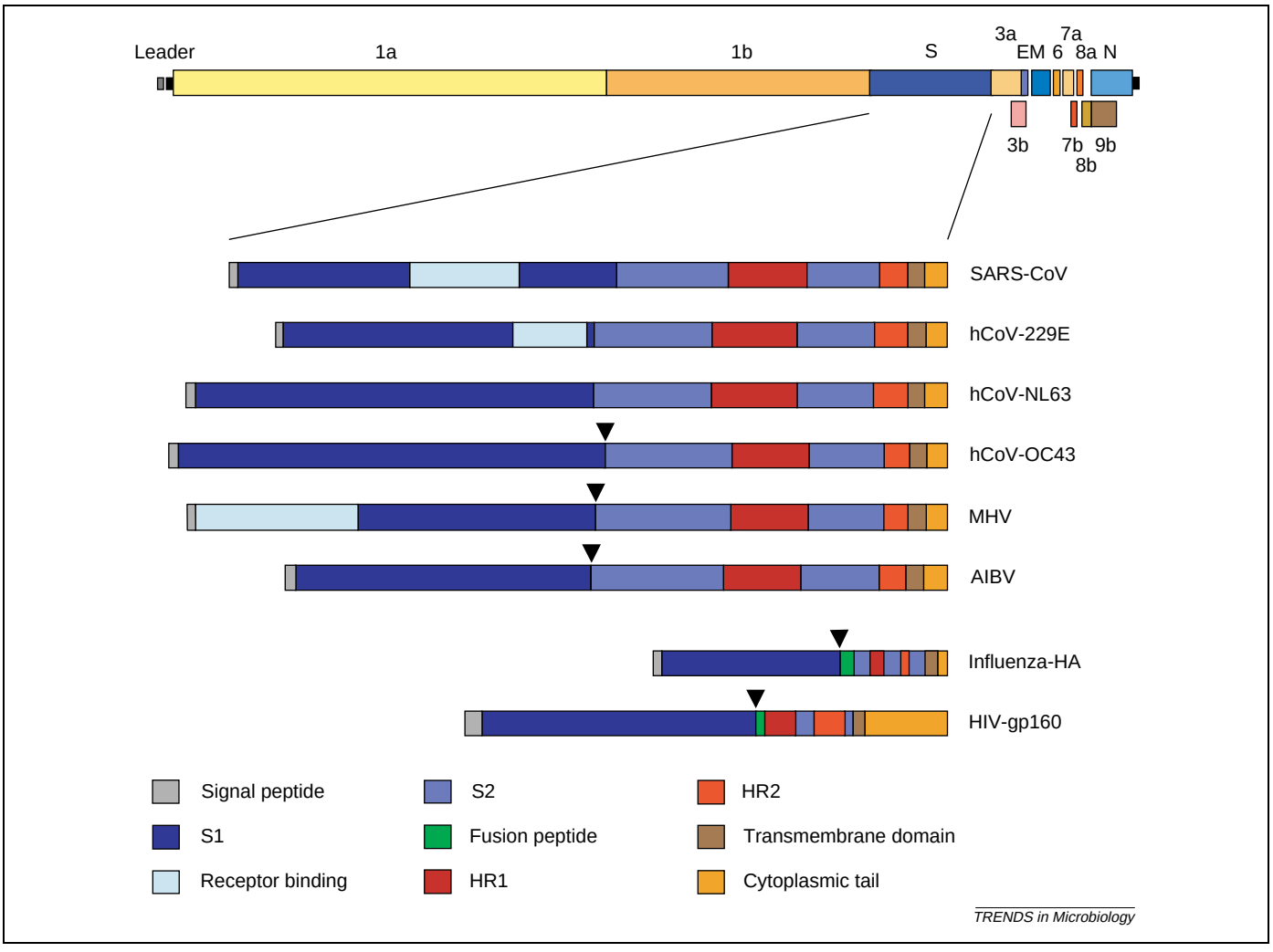

Figure 1. Structural features of the coronavirus (CoV) spike (S)-protein. The localization of the S-gene within the SARS-CoV genome is shown in the upper panel [7,8]. S-proteins of coronaviruses exhibit a domain organization that is characteristic of class I fusion proteins. Prototype members of this group of proteins include hemagglutinin (HA) of influenza virus and the gp160 envelope protein of human immunodeficiency virus (HIV) [49], which are also shown. Generally, these viral glycoproteins can be subdivided into the N-terminal receptor-binding S1 subunit, followed by the S2 subunit containing structural elements required for membrane fusion: a fusogenic peptide and two amino acid stretches with helical symmetry (HR1 and HR2) [17,50]. Proteolytic cleavage into the S1 and S2 subunits by host-cell proteases is indicated by a triangular arrow. Functional regions within the NL63 S-protein were predicted using sequence similarity to the closest related coronavirus member hCoV-229E [61]. Abbreviations: AIBV, avian infectious bronchitis virus; hCoV, human CoV; HR, heptad repeat; MHV, murine hepatitis virus; SARS, severe acute respiratory syndrome.

sequence conservation is found in heptad repeats (HRs) located within the S2 regions [7,8], underlining their important function (Table 1). All CoV S-proteins contain an N-terminal signal peptide, which facilitates transport into the endoplasmatic reticulum, where the proteins are extensively glycosylated. Notably, SARS-CoV S harbors 23 consensus sites for N-linked glycosylation [7,8].

Box 1. Replication of coronaviruses

The spike protein (S) of coronavirus (CoV) particles binds to a cellular receptor that promotes fusion of the viral and cellular membranes. Thereby, the positive-stranded RNA genome is released into the cytoplasm, where the host cell machinery immediately translates the first large overlapping open-reading frames ORF1a/b. The resulting protein, the viral RNA-dependent RNA-polymerase, is responsible for replication of the viral genome as well as for generation of a set of subgenomic mRNAs. These comprise RNAs encoding the nucleocapsid protein (N) and the envelope glycoproteins E (small envelope protein), M (membrane protein), the S-protein and 8 proteins of unknown function. The envelope proteins are processed in the ER–Golgi apparatus and transported to the budding compartment, where the M-protein associates with the helical nucleocapsid and the E- and S-proteins. Progeny virions are released from infected cells by exocytosis.

Dendritic cells transmit SARS-CoV to target cells

Binding of viral GPs to cellular factors other than the receptor(s) does not enable entry but can enhance viral infection. Therefore, various pathogens, including HIV, are thought to interact with factors on dendritic cells (DCs) to promote their spread within infected individuals [12]. Binding of HIV to DCs facilitates infection of nearby susceptible cells through a mechanism that is not completely understood. The lectin DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin) or related molecules might be instrumental to this process because DC-SIGN expressed on cell lines binds to the GP of HIV and catalyzes infection of adjacent receptor-positive cells [12]. DC-SIGN also interacts with SARS-CoV S-protein and augments infection with retroviral particles bearing the S-protein in their envelope [13,14]. This observation is reflected by efficient DC-mediated

Table 1. Characteristics of coronavirus spike proteins[a]

Spike[b]	Group	Cleavage[c]	% identity[d]	% identity HR1	% identity HR2	Receptor[e]	RBD[f]
hCoV-229E	1	–	20.8	43	20	hAPN	417–547
NL63	1	?	20.2	43.3	20	?	?
hCoV-OC43	2	+/–	25.5	55.9	38.8	Neu5,9Ac2	?
MHV	2	+	24.9	53.7	36.7	CEACAM1	1–330
SARS-CoV	2	–	100	100	100	ACE-2	318–510
AIBV	3	+	21.8	44.1	38.8	feAPN	?

[a]Abbreviations: ACE-2, angiotensin-converting enzyme 2; AIBV, avian infectious bronchitis virus; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; feAPN, feline aminopeptidase N; hAPN, human aminopeptidase N; HR, heptad repeat; MHV, murine hepatitis virus; Neu5 9Ac2, 5-N-acetyl-9-O-acetylsialic acid; RBD, receptor-binding domain; SARS-CoV, severe acute respiratory syndrome coronavirus.
[b]GenBank accession numbers: hCoV-229E, P15423; hCoV-NL63, YP_003767; hCoV-OC43, AAR 01015; MHV, AAR92028; SARS-CoV, AAP33697; AIBV, P12651.
[c]See Refs. [17,25,45,46,48].
[d]Amino acid identities were determined by pairwise sequence alignment with SARS-CoV spike set as 100%. The HR1 and HR2 regions were taken from Ref. [45].
[e]See Refs. [17,21,22].
[f]See Refs. [16,18,23,25].

SARS-CoV transmission to target cells [13,14], however, factors other than DC-SIGN are clearly involved in viral transfer [13]. Notably, DCs are not permissive to virus infection, indicating that productive infection is not required for transmission [13,14]. However, the interaction of SARS-CoV with DCs could contribute to SARS pathogenesis. Attachment of SARS-CoV to dermal DCs might facilitate viral spread in the skin, whereas DC-SIGN-positive alveolar macrophages could promote SARS-CoV replication in the lung. Moreover, internalization of SARS-CoV by DCs might provide the virus with a means of immune escape.

Angiotensin-converting enzyme 2 is a major receptor for SARS-CoV

Although DCs can enhance viral infection using attachment factors, such as DC-SIGN, engagement of cellular receptors is required for infectious entry into the host cell. The first essential step in CoV S-driven infection is therefore the interaction of the S-protein with cellular receptors. Consequently, the S-protein ectodomain is the sole determinant of CoV cell-tropism [15]. The receptor-binding domain located in the S1 subunit exhibits little conservation between CoVs, reflecting the interaction with different cellular receptors (Figure 1; Table 1). Thus, residues 407 to 547 in the S-protein of 229E interact with the metalloprotease CD13 [16,17], whereas residues 1 to 330 of the murine hepatitis virus (MHV) S-protein bind to carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) [17,18]. Species-specific differences in the primary sequence of their cognate receptors can determine the host range of these CoVs [19,20], highlighting the specificity of S-protein interactions with cellular receptors.

Because of the multiple implications for cell tropism, host range and inhibitor development, efforts from several laboratories focused on the identification of the cellular receptors for SARS-CoV. In November 2003, Li *et al.* [21] reported that the metallopeptidase angiotensin-converting enzyme 2 (ACE2) is a receptor for SARS-CoV. They had used a soluble S1–immunoglobulin (Ig) fusion protein for immunoprecipitation experiments with lysates from Vero E6 cells, the cell type used for the isolation of SARS-CoV. Subsequent proteomic analysis revealed ACE2 to be a high-affinity binding partner of S1. Inhibition of SARS-CoV infection of susceptible cells using antibodies against ACE2, in conjunction with the observation that ACE2 expression in an otherwise poorly susceptible cell line strongly augmented viral replication, indicated that the protein facilitated SARS-CoV infection [21]. Wang *et al.* [22] also identified ACE2 as a SARS-CoV receptor by using a different approach.

Binding studies using soluble fragments of SARS-CoV S-protein revealed residues 318 to 510 to be the minimal receptor-binding domain [23–25]. An initial search for S-protein residues that are important for ACE2 binding pointed to E452 and D454, with the latter being crucial for association with ACE2 [24]. However, the exact regions of ACE2 that are in contact with the S-protein remain undetermined. Recently, the structure of the ACE2 ectodomain has been resolved, revealing two ridges flanking the catalytic site [26]. Molecular modeling suggests that these ridges might interact with the S-protein [27]. Interestingly, binding of ACE2 to an inhibitor and probably also to substrate induces structural changes within these ridges [26] and might interfere with binding to the S-protein. Detailed analysis will be required to identify residues within the S-protein and ACE2 that are critical for their interaction; and therefore represent attractive targets for inhibitors. These studies might also have important implications for the development of small animal models. Thus, ACE2 from African green monkeys enables efficient entry of SARS-CoV [28], and infection of some macaque species reproduces aspects of SARS in humans [5,6], while viral replication in mice is less robust and does not induce disease [29]. It will therefore be important to determine if a potentially reduced interaction of SARS-CoV S-protein with murine ACE2 limits viral spread in these animals. If so, the generation of transgenic animals is warranted [21].

ACE2, a carboxypeptidase that cleaves polypeptides from the renal-angiotensin system [30], is essential for cardiac function [31] and is expressed in various tissues and organs [32]. Importantly, major target cells of SARS-CoV, such as pneumocytes [4,33–35], express ACE2 [32], and expression in cell lines correlates with permissiveness to SARS-CoV S-driven infection [28], indicating that ACE2 plays a central role in SARS-CoV replication. Notably, evidence for the requirement of a coreceptor or the existence of alternative receptors for SARS-CoV entry into certain tissues has not been reported to date. However, ACE2-dependent infection of organs other

than the lung might contribute to SARS pathogenesis. For example, small intestinal enterocytes express ACE2 [32] and are permissive for SARS-CoV [35–37]. Moreover, the efficient infection of renal epithelial cells of different species [14] and isolation of the virus from kidney tissue of a SARS patient [3] suggests that SARS-CoV infection of kidney cells might contribute to acute renal failure observed in some SARS patients [14,38]. Infection of intestinal enterocytes and kidney cells could facilitate viral transmission via feces. Viral RNA has been detected in stool samples from SARS patients [4,37,39], however, it is unclear if transmission via feces promoted viral spread during the 2003 outbreak. Hepatocytes from SARS patients were also found to be infected [36], and some ACE2-expressing hepatoma cell lines are highly permissive to replication of SARS-CoV [14,28,40,41]. Infection of hepatocytes might therefore partially account for the altered levels of liver-specific enzymes commonly observed in SARS patients [4,40]. Because liver tissue has been found to be largely negative for ACE2 protein expression [32], it will be interesting to examine if viral entry is facilitated by low levels of ACE2 or different factors.

SARS-CoV S triggering: low pH versus receptor engagement

Receptor engagement can activate the fusion machinery of a viral GP in two ways (Figure 2). First, binding to receptor can directly activate the fusion process [42,43], which is the case for HIV and murine leukemia virus (MLV) GPs. Alternatively, receptor engagement can trigger the internalization of viral particles into endosomes where protonation activates GP-driven membrane fusion [42,43]. Influenza hemagglutinin and the vesicular stomatitis virus G-protein (VSV-G) are activated by low pH. Inhibitors of vacuolar acidification block infection by S-bearing pseudotypes, indicating that low pH triggers the fusion activity of SARS-CoV S-protein (Figure 2) [14,40,41]. However, SARS-CoV S-driven cell-to-cell fusion can occur in the absence of low pH [25,44], at least under conditions of GP and receptor overexpression. Therefore, the S-protein of SARS-CoV might be able to mediate membrane fusion in a pH-dependent and independent fashion, and several parameters might control which stimulus is required.

One such parameter could be the association between the S1 and S2 subunits of the SARS-CoV S-protein. Many class I fusion proteins are cleaved into an outer and a transmembrane subunit by cellular proteases and cleavage is essential for function. By contrast, S-proteins of group I CoVs are not cleaved at all and cleavage of the S-protein of MHV, a group II CoV, appears to be cell-type dependent and not required for function [45,46]. No obvious consensus sites for cellular proteases were detected in SARS-CoV S [7,8] and efficient cleavage of the protein has not been reported [25,45,47,48], although more cell types need to be examined. Notably, protease

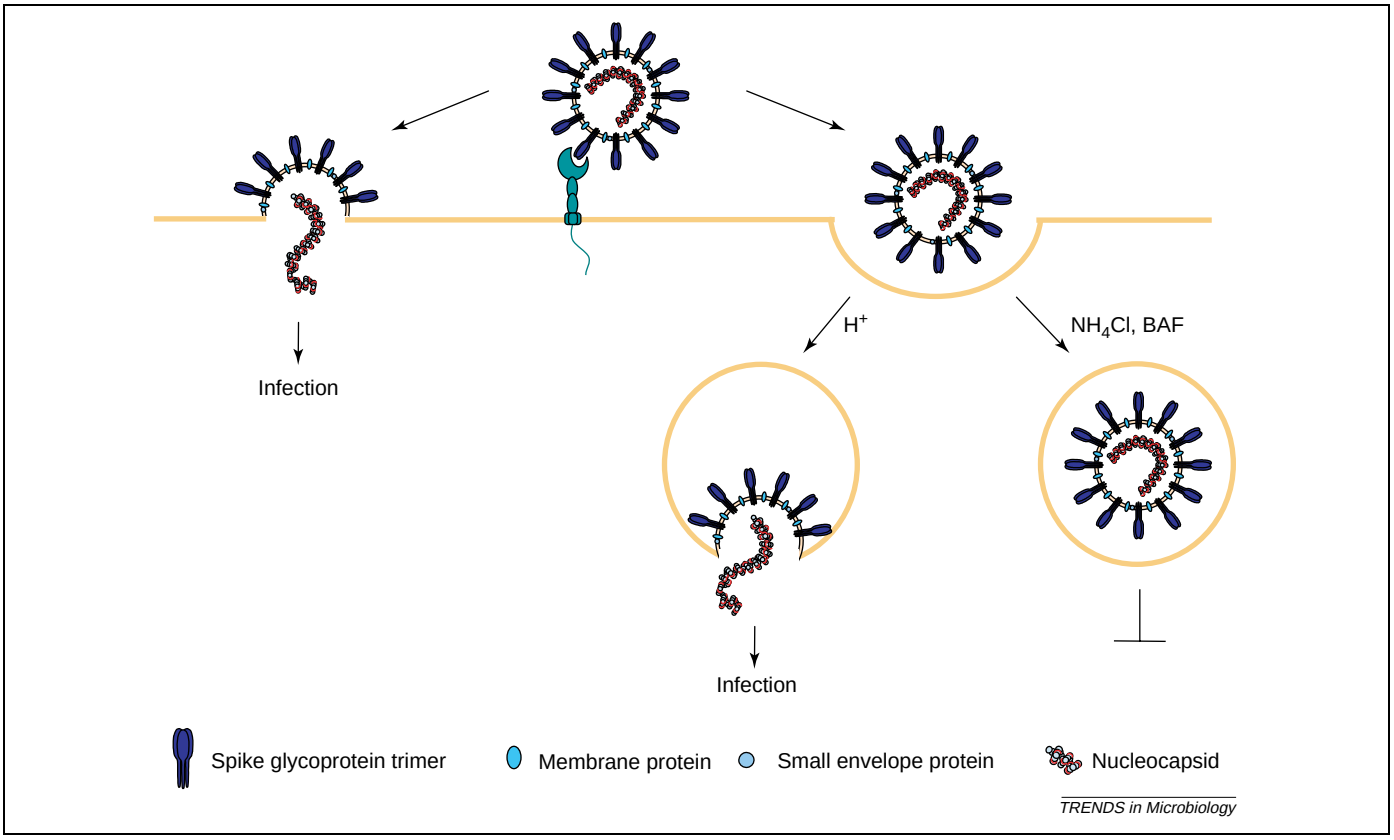

Figure 2. Routes of virus entry into target cells. Binding of a viral glycoprotein (GP) to a receptor can induce conformational alterations, which facilitate fusion of the viral and plasma membrane leading to release of the viral nucleocapsid into the cytoplasm (left). Alternatively, binding to the receptor can be followed by uptake of the virion into an endosomal compartment (right) [42,43]. Proton influx into the endosome can then trigger the membrane fusion activity in GP. Inhibitors of acidification, such as ammonium chloride (NH_4Cl) or bafilomycin A (BAF), can be used to dissect both pathways because they specifically block pH-dependent membrane fusion. For entry of SARS-CoV S-bearing particles, the endosomal pathway appears to be important [14,40,41]. However, an acid environment has been shown to be dispensable for the fusion of cells that express the S-protein to adjoining cells that express the SARS-CoV receptor ACE2 (angiotensin-converting enzyme 2) [25,41].

treatment of S-expressing cells increased cell-to-cell fusion [41], indicating that cleavage of S might enable pH-independent, receptor-dependent triggering of the fusion activity. The trigger for SARS-CoV S-driven membrane fusion and the possible cleavage by cellular proteases await further investigation.

Membrane fusion and its inhibition

Two functional elements located in the transmembrane subunit of CoV S-proteins are pivotal to membrane fusion: a putative fusion peptide and two HRs (Figure 3). The function of these elements has been elucidated in the context of prototype class I fusion proteins, such as HIV gp160 [49]. Cleavage of gp160 produces the fusion active form of the transmembrane subunit gp41, which is oriented perpendicular to the viral membrane and contains a fusion peptide (a stretch of hydrophobic amino acids) at its N-terminus. Two HRs (HR1 and HR2) are located between the fusion peptide and the transmembrane domain. During the fusion process, the fusion peptide inserts into the target cell membrane, HR2 folds back onto HR1, and a six-helix bundle structure (trimer of dimers) is formed. In this conformation, the HRs are oriented in an anti-parallel fashion, thereby bringing the fusion peptide (inserted into the target cell membrane) and the transmembrane domain (inserted into the viral membrane) into close contact, which ultimately promotes membrane fusion [49].

Bosch *et al.* [50] demonstrated that fusion driven by the S-protein of MHV follows similar principles, however, a major difference was noted. Cleavage of many CoV S-proteins, including that of SARS-CoV, is dispensable for function and therefore these proteins must harbor an internal fusion peptide similar to the G-protein of VSV. A computer-based analysis has predicted a potential fusion peptide at the N-terminus of HR1 in SARS-CoV S [45,51]. In the light of these observations, the model for membrane fusion illustrated previously must be revised for the SARS-CoV S-protein. Thus, upon exposure to low pH it is probable that an internal fusion peptide, which is covalently associated with both the S1 and S2 subunits, inserts into the target cell membrane, and membrane fusion is driven by the formation of the six-helix bundle between HR1 and HR2 (Figure 3). In case of influenza HA, a low pH environment triggers irreversible conformational changes associated with membrane fusion, whereas exposure of VSV-G to low pH induces a reversible transition into the fusion active state [49]. The nature of pH-induced conformational changes in SARS-CoV S-protein remains to be determined.

If the model proposed above accurately describes SARS-CoV S-driven membrane fusion, one would expect that peptides mimicking HR1 or HR2 should assemble into a six-helix bundle and that such peptides would inhibit SARS-CoV S-mediated membrane fusion. The latter speculation is based on evidence obtained with several viral class I fusion proteins, including MHV S-protein [50], for which HR-derived peptides were shown to inhibit fusion by preventing the formation of the six-helix bundle [49]. The peptide T20, which potently inhibits

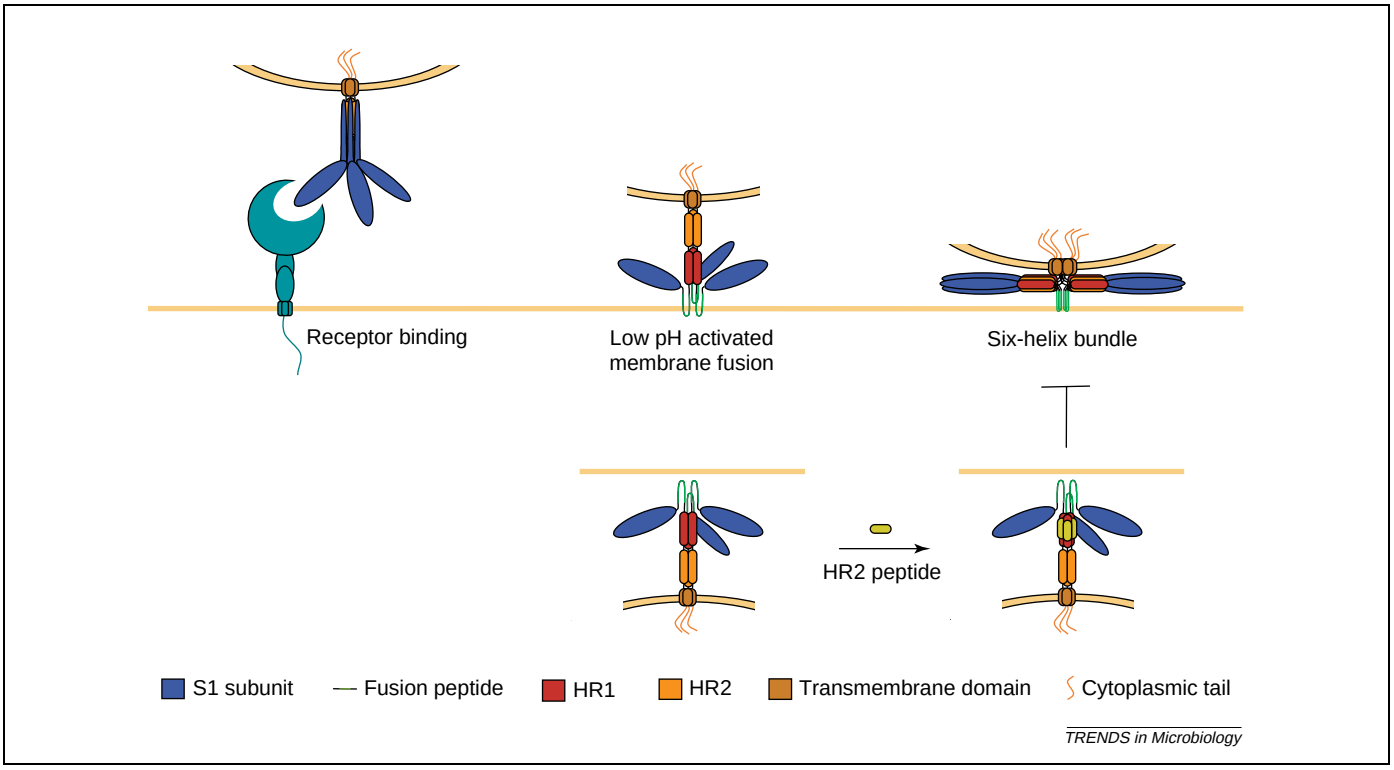

Figure 3. Membrane fusion and its inhibition. Binding of SARS-CoV spike (S)-protein to ACE2 (angiotensin-converting enzyme 2) promotes internalization into endosomes, where the low pH environment triggers fusion activity in the S-protein. Membrane fusion is driven by the S2 subunit of S, which contains a putative fusion peptide and two heptad repeats (HRs) [7,8]. Upon insertion of the fusion peptide into the host cell membrane, HR1 folds back onto HR2, forming a highly stable six-helix bundle [45,47,51,54]. The formation of the six helix bundle brings the viral and the host cell membrane in close proximity and ultimately promotes membrane fusion (upper panel). The HRs are attractive targets for antiviral therapy; synthetic peptides mimicking HR2 can complex the HR1 regions, which inhibits the formation of the six-helix bundle (lower panel) [45,47,53].

HIV gp160-driven membrane fusion when present in the low-nanomolar range, has been approved for use in patients, constituting the first member of entry inhibitors, a new class of therapeutics [52].

Several recent studies have indeed reported that SARS-CoV S-mediated fusion can be inhibited by HR2 but not HR1-derived peptides [45,47,53], which in general reflects the situation observed with HIV [49]. Mixing SARS-CoV S HR1 and HR2 peptides resulted in the formation of a six-helix bundle, in which the HR1 and HR2 peptides were oriented in an antiparallel fashion [45,47,51,54]. Residues 916–950 in HR1 and 1151–1185 in HR2 were found to be crucial for the interaction [47,51]. With one exception [53], micromolar concentrations of the peptides were required for efficient inhibition of viral infection [45,47], indicating that although these peptides are effective, further optimization is required to achieve efficient inhibition of SARS-CoV in infected individuals.

Therapeutic approaches that target SARS-CoV entry are not limited to impeding the membrane fusion reaction. A polypeptide derived from the S1 portion of SARS-CoV S has been shown to block infection, possibly by occupying the S-binding site in ACE2 [24]. Moreover, the purified ACE2 ectodomain specifically inhibited S-driven infection [28], and it needs to be assessed if smaller portions of ACE2 also exhibit blocking activity. The pending identification of the S-binding site in ACE2 will greatly facilitate these studies.

S-based vaccines: successes and pitfalls

The CoV S-proteins are the major target of the humoral and cellular immune response and constitute prime candidates for vaccine development. Vaccination of mice with SARS-CoV S-encoding DNA triggered a humoral and cellular immune response and induced protection against infection [55]. Depletion of T cells and Ig transfer experiments revealed that protection was due to neutralizing antibodies [29,55]; protection can be achieved by the sole administration of S-specific neutralizing monoclonal antibodies [56]. Production of neutralizing antibodies was also observed upon immunization of macaques with adenovirus [57] and mice with vaccinia virus [48], both encoding the S-protein, and in the latter case protection of the vaccinated animals against SARS-CoV was demonstrated. These data, in conjunction with neutralization of S-driven infection using sera obtained from convalescent SARS patients [40,41] and also using a monoclonal antibody directed against the S1 subunit [58], indicate that S-based vaccines are promising for future human trials. However, triggering inflammation in S-vaccinated but not in control animals has also been reported [59] and an enhancing effect of S-specific antibodies on feline infectious peritonitis virus replication is well established [60]. Therefore, S-based vaccines need to be carefully characterized in several animal models before being administered to humans.

Concluding remarks

ACE2 is a major receptor for SARS-CoV, and its interaction with the S-protein can be targeted by therapeutics and vaccines. SARS-CoV S-protein is a class I fusion protein that can be inhibited by strategies known to be effective against related viral GPs. Fusion inhibitors are already available and will be complemented by compounds that target different steps of SARS-CoV entry. Vaccines designed on the basis of the S-protein are promising, but require thorough evaluation in various animal models before administration to humans. Future studies will reveal detailed insights on the structural organization and membrane fusion activity of the S-protein, which in turn will facilitate the generation of more potent inhibitors and vaccines candidates.

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