CHAPTER TWO

Coronavirus Spike Protein and Tropism Changes

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Contents

Abstract

Coronaviruses (CoVs) have a remarkable potential to change tropism. This is particularly illustrated over the last 15 years by the emergence of two zoonotic CoVs, the severe acute respiratory syndrome (SARS)- and Middle East respiratory syndrome (MERS)-CoV. Due to their inherent genetic variability, it is inevitable that new cross-species transmission events of these enveloped, positive-stranded RNA viruses will occur. Research into these medical and veterinary important pathogens—sparked by the SARS and MERS outbreaks—revealed important principles of inter- and intraspecies tropism changes. The primary determinant of CoV tropism is the viral spike (S) entry protein. Trimers of the S glycoproteins on the virion surface accommodate binding to a cell surface receptor and fusion of the viral and cellular membrane. Recently, high-resolution structures of two CoV S proteins have been elucidated by single-particle cryo-electron microscopy. Using this new structural insight, we review the changes in the S protein that relate to changes in virus tropism. Different concepts underlie these tropism changes at the cellular, tissue, and host species level, including the promiscuity or

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adaptability of S proteins to orthologous receptors, alterations in the proteolytic cleavage activation as well as changes in the S protein metastability. A thorough understanding of the key role of the S protein in CoV entry is critical to further our understanding of virus cross-species transmission and pathogenesis and for development of intervention strategies.

1. INTRODUCTION

Coronaviruses (CoVs) (order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*) are enveloped, positive-sense RNA viruses that contain the largest known RNA genomes with a length of up to 32 kb. The subfamily *Coronavirinae*, which contains viruses of both medical and veterinary importance, can be divided into the four genera *alpha*-, *beta*-, *gamma*- and *deltacoronavirus* (α-, β-, γ- and δ-*CoV*). The coronavirus particle comprises at least the four canonical structural proteins E (envelope protein), M (membrane protein), N (nucleocapsid protein), and S (spike protein). In addition, viruses belonging to lineage A of the *betacoronaviruses* express the membrane-anchored HE (hemagglutinin–esterase) protein. The S glycoprotein contains both the receptor-binding domain (RBD) and the domains involved in fusion, rendering it the pivotal protein in the CoV entry process.

Coronaviruses primarily infect the respiratory and gastrointestinal tract of a wide range of animal species including many mammals and birds. Although individual virus species mostly appear to be restricted to a narrow host range comprising a single animal species, genome sequencing and phylogenetic analyses testify that CoVs have crossed the host species barrier frequently (Chan et al., 2013; Woo et al., 2012). In fact most if not all human coronaviruses seem to originate from bat CoVs (BtCoVs) that transmitted to humans directly or indirectly through an intermediate host. It therefore appears inevitable that similar zoonotic infections will occur in the future.

In the past 15 years, the world witnessed two such zoonotic events. In 2002–2003 cross-species transmissions from bats and civet cats were at the base of the SARS (severe acute respiratory syndrome)-CoV epidemic that found its origin in the Chinese Guangdong province (Li et al., 2006; Song et al., 2005). The SARS-CoV nearly became a pandemic and led to over 700 deaths, before it disappeared when the appropriate hygiene and quarantine precautions were taken. In 2012, the MERS (Middle East respiratory syndrome)-CoV emerged in the human population on the Arabian

Peninsula and currently continues to make a serious impact on the local but also global health system with 1800 laboratory confirmed cases and 640 deaths as of September 1, 2016 (WHO | Middle East respiratory syndrome coronavirus (MERS-CoV) – Saudi Arabia, 2016). The natural reservoir of MERS-CoV is presumed to be in dromedary camels from which zoonotic transmissions repeatedly give rise to infections of the lower respiratory tract in humans (Alagaili et al., 2014; Azhar et al., 2014; Briese et al., 2014; Reusken et al., 2013; Widagdo et al., 2016). Besides these two novel CoVs, four other CoVs were previously identified in humans which are found in either the *alphacoronavirus* (HCoV-NL63 and HCoV-229E) or the *betacoronavirus* genera (HCoV-OC43 and HCoV-HKU1). Phylogenetic analysis has shown that the bovine CoV (BCoV) has been the origin for HCoV-OC43 following a relatively recent cross-species transmission event (Vijgen et al., 2006). Moreover, HCoV-NL63, HCoV-229E, SARS-CoV, and MERS-CoV also have been predicted to originate from bats (Annan et al., 2013; Bolles et al., 2011; Corman et al., 2015; Hu et al., 2015; Huynh et al., 2012).

In general, four major criteria determine cross-species transmission of a particular virus (Racaniello et al., 2015). The cellular tropism of a virus is determined by the susceptibility of host cells (i.e., presence of the receptor needed for entry) as well as by the permissiveness of these host cells to allow the virus to replicate and to complete its life cycle. A third determinant consists of the accessibility of susceptible and permissive cells in the host. Finally, the innate immune response may restrict viral replication in a host species-specific manner. The above-mentioned criteria may play a critical role in the success of a cross-species transmission event. However, for CoVs, it seems that host tropism and changes therein are particularly determined by the susceptibility of host cells to infection. While CoV accessory genes, including the HE proteins, are thought to play a role in host tropism and adaptation to a new host, the S glycoprotein appears to be the main determinant for the success of initial cross-species infection events. In this review, we focus on the molecular changes in the S protein that underlie tropism changes at the cellular, tissue, and host species level and put these in perspective of the recently published cryo-EM structures.

2. STRUCTURE OF THE CORONAVIRUS S PROTEIN

The CoV S protein is a class I viral fusion protein (Bosch et al., 2003) similar to the fusion proteins of influenza, retro-, filo-, and paramyxoviruses

(Baker et al., 1999; Bartesaghi et al., 2013; Lee et al., 2008; Lin et al., 2014). Like other class I viral fusion proteins, the S protein folds into a metastable prefusion conformation following translation. The size of the abundantly N-glycosylated S protein varies greatly between CoV species ranging from approximately 1100 to 1600 residues in length, with an estimated molecular mass of up to 220 kDa. Trimers of the S protein form the 18–23-nm long, club-shaped spikes that decorate the membrane surface of the CoV particle. Besides being the primary determinant in CoV host tropism and pathogenesis, the S protein is also the main target for neutralizing antibodies elicited by the immune system of the infected host (Hofmann et al., 2004).

The S protein can be divided into two functionally distinct subunits: the globular S_1 subunit is involved in receptor recognition, whereas the S_2 subunit facilitates membrane fusion and anchors S into the viral membrane (Fig. 1A). The S_1 and S_2 domains may be separated by a cleavage site that is recognized by furin-like proteases during S protein biogenesis in the infected cell. X-ray crystal structures of several S domains have furthered our understanding of the S protein in the past. In addition, recent elucidation of the high-resolution structures of the spike ectodomain of two betacoronaviruses—MHV and HCoV-HKU1—by single-particle cryo-electron microscopy (Kirchdoerfer et al., 2016; Walls et al., 2016) has provided novel insights into the architecture of the S trimer in its prefusion state (Fig. 1B and C).

2.1 Structure of the S_1 Subunit

The S_1 subunit of the betacoronavirus spike proteins displays a multidomain architecture and is structurally organized in four distinct domains A–D of which domains A and B may serve as a RBD (Fig. 1C). The core structure of domain A displays a galectin-like β-sandwich fold, whereas domain B contains a structurally conserved core subdomain of antiparallel β-sheets (Kirchdoerfer et al., 2016; Li et al., 2005a; Walls et al., 2016; Wang et al., 2013). Importantly, domain B is decorated with an extended loop on the viral membrane-distal side. This loop may differ greatly in size and structure between virus species of the betacoronavirus genus and is therefore also referred to as hypervariable region (HVR). The cryo-EM structures of the MHV-A59 and HCoV-HKU1 S trimers show an intricate interlocking of the three S_1 subunits (Fig. 1B). Oligomerization of the S protomers results in a closely clustered trimer of the individual B domains close to the threefold axis of the spike on top of the S_2 trimer, whereas the three A domains are

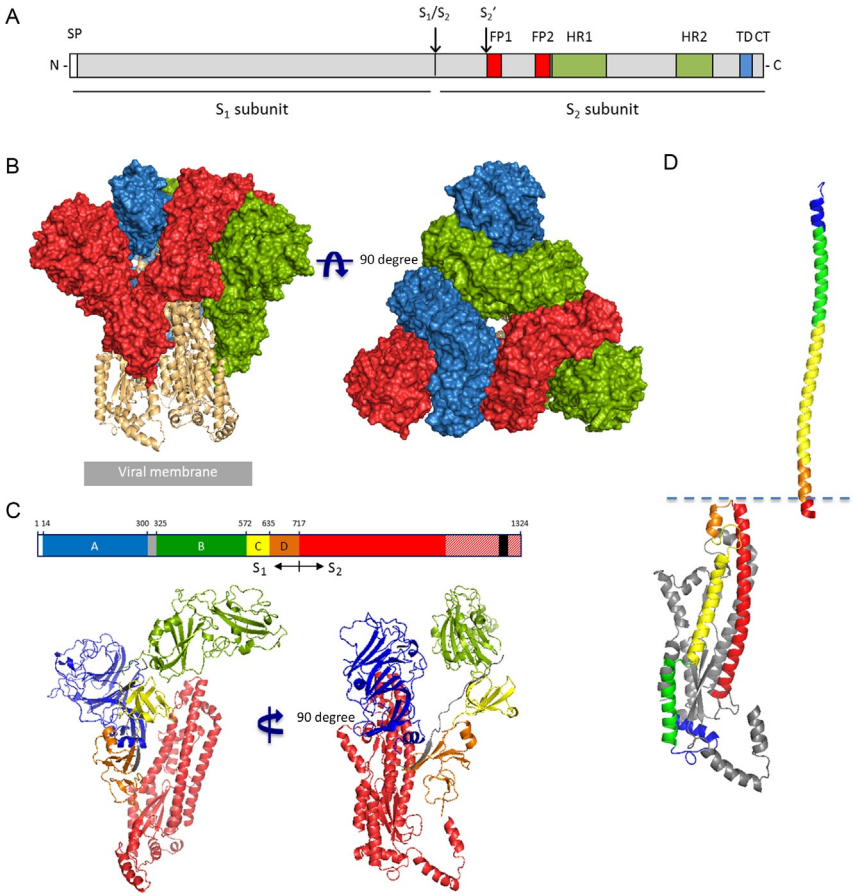

Fig. 1 Spike protein features and structure of the mouse hepatitis coronavirus spike glycoprotein trimer. (A) Schematic linear representation of the coronavirus S protein with relevant domains/sites indicated: signal peptide (SP), two proteolytic cleavage sites (S_1/S_2 and S_2'), two proposed fusion peptides (FP1 and FP2), two heptad repeat regions (HR1 and HR2), transmembrane domain (TD), and cytoplasmic tail (CT). (B) *Front* and *top view* of the trimeric mouse hepatitis coronavirus (strain A59) spike glycoprotein ectodomain obtained by cryo-electron microscopy analysis (Walls et al., 2016; PDB: 3JCL). Three S_1 protomers (surface presentation) are colored in *red*, *blue*, and *green*. The S_2 trimer (cartoon presentation) is colored in *light orange*. (C) Schematic representation of MHV spike protein sequence (drawn to scale), the S_1 domains A, B, C, and D are colored in *blue*, *green*, *yellow*, and *orange*, respectively, and the linker region connecting domains A and B in *gray*, the S_2 region is colored in *red*, and the TM region is indicated as a *black box*. *Red-shaded* region indicates spike region that was

(Continued)

ordered more distally of the center. In contrast to domains A and B, the S_1 C-terminal domains C and D are made up of discontinuous parts of the primary protein sequence and form β-sheet-rich structures directly adjacent to the S_2 stalk core, while the separate S_1 domains are interconnected by loops covering the S_2 surface. Compared to the S_2 subunit, the S_1 subunit displays low level of sequence conversation among species of different CoV genera. Moreover, S_1 subunits vary considerably in sequence length ranging from 544 (infectious bronchitis virus (IBV) S) to 944 (229-related bat coronavirus S) residues in length (Fig. 2), indicating differences in architecture of the spikes of species from different CoV genera. Structural information from the spikes of *gamma-* and *deltacoronavirus* species is currently lacking. Two independently folding domains have been assigned in the S_1 subunit of alphacoronavirus spikes, that can interact with host cell surface molecules, an N-terminal domain (in transmissible gastroenteritis virus (TGEV) S residues 1–245) and a more C-terminal domain (in TGEV S residues 506–655). Contrary to betacoronaviruses, these two receptor-interacting domains in alphacoronavirus spikes are separated in sequence by some 275 residues, which may fold into one or more separate domains. Structural information is only available for the C-terminal S_1 RBD of two α-CoV S proteins, which differs notably from that of betacoronaviruses. The RBD in the S_1 CTR of alphacoronaviruses displays a β-sandwich core structure, whereas a β-sheet core structure is seen for betacoronaviruses (Reguera et al., 2012; Wu et al., 2009).

2.2 Structure of the S_2 Subunit

The highly conserved S_2 subunit contains the key protein segments that facilitate virus-cell fusion. These include the fusion peptide, two heptad

Fig. 1—Cont'd not resolved in the cryo-EM structure. (*Lower panel*) Two views on the structure of the mouse hepatitis virus spike glycoprotein protomer (cartoon representation); domains are colored as depicted earlier. (D) Comparison of the S_2 HR1 region in its pre- and postfusion conformation. (*Lower left*) Structure of the MHV S_2 protomer (cartoon presentation) with four helices of the HR1 region (and consecutive linker region) and the downstream central helix colored in *blue*, *green*, *yellow*, *orange*, and *red*, respectively. (*Upper right*) The structure of a single SARS-CoV S HR1 helix of the postfusion six-helix bundle structure (PDB: 1WYY) is colored according to the homologous HR1 region in the MHV S_2 prefusion structure shown in the *lower left panel*. Structures are aligned based on the N-terminal segment of the central helix (in *red*). Figures were generated with PyMOL.

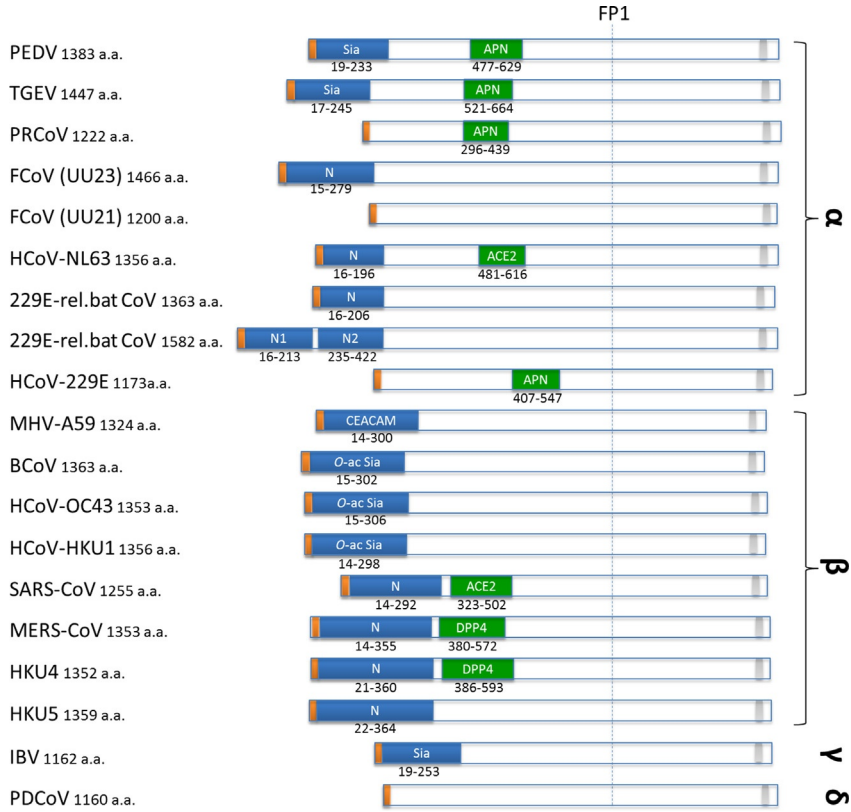

Fig. 2 Overview of currently known receptors and their binding domains within S_1. Schematic representation of coronavirus spike proteins drawn to scale. *Yellow boxes* indicate signal peptides. *Blue boxes* indicate the N-terminal regions in alpha- and betacoronavirus spike proteins, which were mapped based on sequence homology between viruses within the same genus. *Green boxes* indicate known receptor-binding domains in the C-terminal region of S_1. Known receptors are indicated in the *boxes*: *APN*, aminopeptidase N; *ACE2*, angiotensin-converting enzyme 2; *CEACAM*, carcinoembryonic antigen-related cell adhesion molecule 1; *Sia*, sialic acid; *O-ac Sia*, *O*-acetylated sialic acid; *DPP4*, dipeptidyl peptidase-4. *Gray boxes* indicate transmembrane domains. Spikes proteins are shown of PEDV strain CV777 (GB: AAK38656.1), TGEV strain Purdue P115 (GB: ABG89325.1), PRCoV strain ISU-1 (GB: ABG89317.1), Feline CoV strain UU23 (GB: ADC35472.1), Feline CoV strain UU21 (GB: ADL71466.1), Human CoV NL63 (GB: YP_003767.1), 229E-related bat CoV with one N domains (GB: ALK28775.1), 229E-related bat CoV with two N domains (GB: ALK28765.1), Human CoV 229E strain inf-1 (GB: NP_073551.1), MHV strain A59 (GB: ACO72893), BCoV strain KWD1 (GB: AAX38489), HCoV-OC43 strain Paris (GB: AAT84362), HCoV-HKU1 (GB: AAT98580), SARS-CoV strain Urbani (GB: AAP13441), MERS-CoV strain EMC/2012 (GB: YP_009047204), HKU4 (GB: AGP04928), HKU5 (GB: AGP04943), IBV strain Beaudette (GB: ADP06471), and PDCoV

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repeat regions (HR1 and HR2) and the transmembrane domains which are well conserved among CoV species across different genera. In the MHV and HKU1 S prefusion structures, the S_2 domain consists of multiple α-helical segments and a three-stranded antiparallel β-sheet at the viral membrane-proximal end. A 75 Å long central helix located immediately downstream of the HR1 region stretches along the threefold axis over the entire length of the S_2 trimer. The HR1 motif itself folds as four individual α-helices along the length of the S_2 subunit, in contrast to the 120 Å long α-helix formed by this region in postfusion structures (Duquerroy et al., 2005; Gao et al., 2013; Xu et al., 2004). A 55 Å long helix upstream of the S_2' cleavage site runs parallel to and is packed against the central helix via hydrophobic interactions (Fig. 1C). The fusion peptide forms a short helix of which the strictly conserved hydrophobic residues are buried in an interface with other elements of S_2. Unlike other class I fusion proteins, this conserved fusion peptide (FP1) is not directly upstream of HR1 but located some 65 residues upstream of this region (Fig. 1A). Intriguingly, a recent published report provided experimental evidence for the existence of another fusion peptide (FP2) immediately upstream of the HR1 region (Ou et al., 2016), that had been predicted earlier based on the position, hydrophobicity profile and amino acid composition canonical for class I viral fusion peptides (Bosch and Rottier, 2008; Bosch et al., 2004; Chambers et al., 1990). The HR2 region locates closely to the C-terminal end of the S ectodomain, but it appeared to be disordered in both cryo-EM structures and therefore its prefusion conformation remains unknown.

The metastable prefusion conformation of S_2 is locked by the cap formed by the intertwined S_1 protomers. The distal tip of the S_2 trimer connects via hydrophobic interactions with domains B. This distal tip of the S_2 trimer consists of the C-terminal region of HR1 in the prefusion conformation, while the entire HR1 rearranges to form a central three-helix coiled coil in the postfusion structure (Duquerroy et al., 2005; Lu et al., 2014; Supekar et al., 2004). Interactions between this region of the S_2 trimer and domain B may therefore prevent premature conformational changes resulting in the conversion of the prefusion S protein into the very stable

Fig. 2—Cont'd strain USA/Ohio137/2014 (GB: AIB07807). PSI-BLAST analysis using the NTR of the HCoV-NL63 S protein (residues 16–196) as a query detected two homologous regions in the first 425 residues of the 229E-related bat coronavirus spike protein (GB: ALK28765.1)—designated N1 (residues 32–213) and N2 (residues 246–422) with 32% and 35% amino acid sequence identity, respectively, suggesting a duplication of the NTR. Spike proteins are drawn to scale and aligned at the position of the conserved fusion peptide (FP1).

postfusion structure. Also domains C and D of the betacoronavirus S_1 subunit and the linker region connecting domain A and B interact with the surface of the adjacent S_2 protomer and may hence play a role in stabilizing the prefusion S_2 trimer. Domain A appears to play a minor role in this respect in view of its relatively small a surface area that interacts with the S_2 trimer.

3. SPIKE–RECEPTOR INTERACTIONS

3.1 Different Domains Within S_1 May Act as RBD

Over the past decades, molecular studies on the CoV S glycoprotein have shown that both the N-terminal region (NTR, domain A in β-CoV) and the C-terminal region of S_1 (CTR, comprising domain B, C, and D in β-CoV) can bind host receptors and hence function as RBDs (Fig. 2) (Li, 2015). The CTR of alpha- and betacoronaviruses appears to bind proteinaceous receptors exclusively. The α-CoV HCoV-229E, serotype II feline CoV (FCoV), TGEV, and porcine respiratory coronavirus use the human aminopeptidase N (APN) of their respective hosts as receptors (Bonavia et al., 2003; Delmas et al., 1992; Reguera et al., 2012). The HCoV-NL63 (α-CoV) and SARS-CoV (β-CoV) both utilize angiotensin-converting enzyme 2 (ACE2) as a functional receptor (Li et al., 2005b; Wu et al., 2009), whereas the β-CoVs MERS-CoV and BtCoV-HKU4 recruit dipeptidyl peptidase-4 (DPP4) as a functional receptor (Lu et al., 2013; Mou et al., 2013; Raj et al., 2013; Wang et al., 2014; Yang et al., 2014).

The receptor-binding motifs (RBMs) in the S_1 CTRs of alpha- and betacoronavirus spike proteins are presented on one or more loops extending from the β-sheet core structure. Within *alpha-* and *betacoronavirus* genera the RBD core is structurally conserved yet the RBM(s) that determine receptor specificity may vary extensively. For instance, the CTR of the α-CoVs PRCoV and HCoV-NL63 has a similar core structure suggesting common evolutionary origin but diverged in their RBMs recruiting different receptors (APN and ACE2, respectively). A similar situation is seen for the CTRs of β-CoVs SARS-CoV and MERS-CoV that bind ACE2 and DPP4, respectively (Li, 2015). Conversely, the CTRs of the α-CoV HCoV-NL63 and β-CoV SARS-CoV both recognize ACE2, yet via distinct molecular interactions (ACE2 recognition via three vs one RBM, respectively), which suggested a convergent evolution pathway for these viruses in recruiting the ACE2 receptor (Li, 2015). The core

structures of the CTRs in α- and β-CoVs provide a scaffold to present RBMs from extending loop(s), which may accommodate facile receptor switching by subtle alterations in or exchange of the RBMs via mutation/recombination.

Contrary to the CTR, the NTR appears to mainly bind glycans. The NTR of the α-CoV TGEV and of the γ-CoV IBV S proteins binds to sialic acids (Promkuntod et al., 2014; Schultze et al., 1996), while the NTR of betacoronaviruses including BCoV and HCoV-OC43 was shown to bind to *O*-acetylated sialic acids (Künkel and Herrler, 1993; Peng et al., 2012; Schultze et al., 1991; Vlasak et al., 1988). Only the NTR of MHV (domain A) is known to interact with a protein receptor, being mCEACAM1a (Peng et al., 2011), while lacking any detectable sialic acid-binding activity (Langereis et al., 2010). However, as the NTR of MHV displays the β-sandwich fold of the galectins, a family of sugar-binding proteins, it probably has evolved from a sugar-binding domain (Li, 2012).

The presence of RBDs in different domains of the S protein that can bind either proteinaceous or glycan receptors illustrates a functional modularity of this glycoprotein in which different domains may fulfill the role of binding to cellular attachment or entry receptors. The CoV S protein is thought to have evolved from a more basic structure in which receptor recognition was confined to the CTR within S_1 (Li, 2015). The observed deletions of the NTR in some CoV species in nature are indicative of a less stringent requirement and integration of this domain with other regions of the spike trimer compared to the more C-terminally located domains of S_1 and support a scenario in which the NTR has been acquired at a later time point in CoV evolutionary history. For example, the NTR of MHV, which displays a human galectin-like fold, was suggested to originate from a cellular lectin acquired early on in CoV evolution (Peng et al., 2011). Acquisition of glycan-binding domains and fusion thereof to the ancestral S protein may have resulted in a great extension of CoV host range and may have caused an increase in CoV diversity. The general preference of the NTR and CTR to bind to, respectively, glycan or protein receptors may be related to their arrangement in the S protein trimer. In contrast to the CTR, which is located in the center of the S trimer, the NTR is more distally oriented (Fig. 1B). As protein–glycan interactions are often of low affinity, the more distal orientation of domain A may allow multivalent receptor interactions, thereby increasing avidity. Interestingly, some CoVs appear to have a dual receptor usage as they may bind via their NTR and CTR to glycan and protein receptors, respectively (Fig. 2).

3.2 CoV Protein Receptor Preference

Although the number of currently known CoV receptors is limited, receptor usage does not appear to be necessarily conserved between closely related virus species such as HCoV-229E (APN) and HCoV-NL63 (ACE2), whereas identical receptors (ACE2) can be targeted by virus species from different genera such as HCoV-NL63 and SARS-CoV. It seems that CoVs prefer certain types of host proteins as their entry receptor, with three out of four of the so far identified proteinaceous receptors being ectopeptidases (APN, ACE2, and DPP4), although enzymatic activity of these proteins was shown not to be required for infection by their respective viruses (Bosch et al., 2014). Possibly, the localization to certain membrane microdomains and efficient internalization of two of these proteins in polarized cells (APN and DPP4) may contribute to their suitability to function as entry receptors (Aït-Slimane et al., 2009). In the case of MERS-CoV, the region of DPP4 that is bound by the S protein coincides with the binding site for its physiological ligand adenosine deaminase (Raj et al., 2014). Employment of conserved epitopes such as these may also contribute to the cross-species transmission potential of viruses (Bosch et al., 2014), as is exemplified by MERS-CoV being able to use goat, camelid, cow, sheep, horse, pig, monkey, marmoset, and human DPP4 as entry receptor (Barlan et al., 2014; Eckerle et al., 2014; Falzarano et al., 2014; Müller et al., 2012; van Doremalen et al., 2014). Similarly, this may apply for the ability of feline, canine, porcine, and human CoVs to use fAPN as entry receptor, at least in vitro (Tresnan et al., 1996).

4. S PROTEIN PROTEOLYTIC CLEAVAGE AND CONFORMATIONAL CHANGES

Coronavirus entry is a tightly regulated process that appears to be orchestrated by multiple triggers that include receptor binding and proteolytic processing of the S protein and that ultimately results in virus-cell fusion. It is initiated by virion attachment mediated through interaction of either the NTR or CTR (or both) in the S_1 subunit of the spike protein with host receptors. Upon attachment, the virus is taken up via receptor-mediated endocytosis by clathrin- or caveolin-dependent pathways (Burkard et al., 2014; Eifart et al., 2007; Inoue et al., 2007; Nomura et al., 2004) although other entry routes have also been reported (Wang et al., 2008). Prior to and/or during endocytic uptake the CoV S protein

is proteolytically processed. The spike protein may contain two proteolytic cleavage sites. One of the cleavage sites is located at the boundary between the S_1 and S_2 subunits (S_1/S_2 cleavage site), while the other cleavage site is located immediately upstream of the first fusion peptide (S_2' cleavage site). Although not irrevocably proven, it is expected that all CoVs depend on proteolytic cleavage on or close to S_2' for fusion to occur. Virus-cell fusion thus not only critically depends on the conformational changes following spike–receptor engagement, and perhaps on acidification of endosomal vesicles (Eifart et al., 2007; Matsuyama and Taguchi, 2009; Zelus et al., 2003), but also on proteolytic activation of the S protein by proteases along the endocytic route (Burkard et al., 2014; Simmons et al., 2005). Indeed, inhibition of intracellular proteases has been shown to block virus entry and virus-cell fusion (Burkard et al., 2014; Frana et al., 1985; Simmons et al., 2005; Yamada and Liu, 2009). The specific proteolytic cleavage requirements of the S protein at the S_1/S_2 boundary and particularly at the S_2' site may furthermore determine the intracellular site of fusion (Burkard et al., 2014). In agreement herewith, it has become evident that the protease expression profile of host cells may form an additional determinant of the host cell tropism of coronaviruses (Millet and Whittaker, 2015).

Analysis of the CoV S prefusion conformation suggests that relocation (or shedding) of the S_1 subunits that cap the S_2 subunit is a prerequisite for the conformational changes in S_2 that ultimately result in fusion. Shedding of S_1 probably requires receptor binding as well as proteolytic processing at S_1/S_2. The cryo-EM structure indicates that the S_1/S_2 proteolytic cleavage site is accessible to proteases prior to spike–receptor interaction, and depending on the particular cleavage site present may already be processed in the cell in which the virions are produced. As indicated earlier, the conformational changes in the S protein that result in virus-cell fusion most likely also require cleavage at the S_2' site immediately upstream of the fusion peptide. Interestingly, the S_2' cleavage site is located within an α-helix exposed on the prefusion S structure which prevents efficient proteolytic cleavage (Robertson et al., 2016). This indicates the necessity for preceding conformational changes induced by receptor binding and subsequent shedding of S_1, upon which the secondary structure of the S_2' site transforms into a cleavable flexible loop. Following proteolytic cleavage activation at the S_2' site, hydrophobic interactions between the fusion peptide and the adjacent S_2 helices are disturbed which allows the four α-helices and the connecting regions that make up the HR1 region in the prefusion S protein to refold into a long trimeric coiled coil (Fig. 1D). This coiled coil

forms an N-terminal extension of the central helix projecting the fusion peptide(s) toward the target membrane. Successively, the fusion peptide(s) will be inserted into the limiting membrane of the host cell endocytic compartment. Next, as a consequence of S_2 rearrangements, the two HR regions will interact to form an antiparallel energetically stable six-helix bundle (Bosch et al., 2003, 2004), enabling the close apposition and subsequent fusion of the viral and host lipid bilayers.

5. TROPISM CHANGES ASSOCIATED WITH S PROTEIN MUTATIONS

Changes in the S protein may result in an altered host, tissue, or cellular tropism of the virus. This is clearly exemplified by genomic recombination events that result in exchange of (part of) the S protein and in a concomitant change in tropism. The propensity of CoVs to undergo homologous genomic recombination has been exploited for the genetic manipulation of these viruses (de Haan et al., 2008; Haijema et al., 2003; Kuo et al., 2000). To this end, interspecies chimeric coronaviruses were generated, which carried the spike ectodomain of another CoV and which could be selected based on their altered requirement for an entry receptor. Exchange of S protein genes may also occur in vivo, resulting in altered tropism as is illustrated by the occurrence of serotype II feline infectious peritonitis virus (FIPV). This virus results from a naturally occurring recombination event between feline and canine CoVs (CCoVs) in which the feline virus acquires a CCoV spike gene (Herrewegh et al., 1995; Terada et al., 2014). As a result of the acquisition of this new S protein, the rather harmless enteric feline CoV (FECV) turns into a systemically replicating and deadly FIPV. As FECV has a strict feline tropism (Myrrha et al., 2011), while CCoV has been shown to infect feline cells (Levis et al., 1995), it is likely that serotype II FIPVs arise in cats coinfected with serotype I FECV and CCoV. Furthermore, as different recombination sites have been observed for each serotype II FIPV, while serotype II FECVs have not been observed, it appears that serotype II FIPVs exclusively result of reoccurring recombination events (Terada et al., 2014). In addition to these feline–CCoV recombinants, a chimeric porcine coronavirus with a TGEV backbone and a spike of the porcine epidemic diarrhea virus (PEDV) was recently isolated from swine fecal samples in Italy and Germany, likely also resulting from a recombination event (Akimkin et al., 2016; Boniotti et al., 2016). Moreover, the α-CoV HKU2 BtCoV probably resulted from genomic recombination as it encodes

an S protein that resembles a betacoronavirus S protein except for its N-terminal region that is similar to that of alphacoronaviruses (Lau et al., 2007). Thus, such genomic recombination events are not necessarily restricted to occur between viruses of the same genus.

5.1 S_1 Receptor Interactions Determining Tropism

5.1.1 S_1 NTR Changes

Several changes in the amino-terminal domain of S_1 have been associated with changes in the tropism of the virus. For example, for several α-CoVs, loss of NTR of the S protein appears to be accompanied with a loss of enteric tropism. While the porcine CoV TGEV displays a tropism for both the gastrointestinal and respiratory tract, the closely related PRCoV, which lacks the sialic acid-binding N-terminal region (Krempl et al., 1997), only replicates in the respiratory tract. The loss of sialic acid-binding activity by four-amino acid changes in the NTR of its S protein resulted in an almost complete loss of enteric tropism (Krempl et al., 1997). Similar to TGEV, enteric serotype I FCoVs also have been reported to bind to sialic acids (Desmarets et al., 2014). Large deletions within the S_1 subunit corresponding to the N-terminal region have been found in variants of the systemically replicating FIPV (strains UU16, UU21, and C3663) after intrahost emergence from enteric FECV (Chang et al., 2012; Terada et al., 2012). Also FIPVs seem to have lost the ability to replicate in the enteric tract (Pedersen, 2014). Clinical isolates of human coronavirus 229E as well as of the related alpaca coronavirus, both of which cause respiratory infections, encode relatively short spike proteins that lack the NTR (Crossley et al., 2012; Farsani et al., 2012). In contrast, closely related bat coronaviruses with intestinal tropism contain S proteins with a NTR or sometimes even two copies of the NTR (Corman et al., 2015) (Fig. 2). Overall, these observations suggest that the alphacoronavirus spike NTR—in particular its sialic acid-binding activity—may contribute to the enteric tropism of these alphacoronaviruses, while it is not required for replication in the respiratory tract or in other extraintestinal organs. It has been hypothesized that the sialic acid-binding activity of the spike protein can allow virus binding to (i) soluble sialoglycoconjugates that may protect the virus from hostile conditions in the stomach or (ii) to mucins that may prevent the loss of viruses by intestinal peristalsis and allow the virus to pass the thick mucus barrier, thereby gaining access to the intestinal cells to initiate infection (Schwegmann-Wessels et al., 2003).

Besides deletions of entire domains of the S protein, more subtle changes consisting of amino acid substitutions in S_1 NTR may also suffice to alter the virus' tropism. For example, MHV variants have been observed that acquired the ability to use the human homologue of their murine CEACAM1a receptor to enter cells as a result of mutations in their RBD that is located in S_1 NTR (Baric et al., 1999).

5.1.2 S_1 CTR Changes

As the CTR of the S_1 subunit contains the protein RBD for most CoVs, also mutations in this part of S have been associated with changes in the virus' tropism. Perhaps the most well-known example of viral cross-species transmission involves the SARS-CoV. Studies support a transmission model in which a SARS-like CoV was transmitted from *Rhinolophus* bats to palm civets, which subsequently transmitted the palm civet-adapted virus to humans at local food markets in southern China (Li et al., 2006). According to this model, SARS-like viruses adapted to both the palm civet and human host, which was reflected in the rapid viral evolution observed for these viruses within these species (Song et al., 2005). Two-amino acid substitutions within the RBD were elucidated that are of relevance for binding to the ACE2 proteins of palm civets and humans (Li et al., 2005b, 2006; Qu et al., 2005). From these studies it appears that due to strong conservation of ACE2 between mammalian species only a few amino acid alterations within the RBD are needed to change coronavirus host species tropism. Indeed serial passage of SARS-CoVs in vitro or in vivo can rapidly lead to adaptation to new host species (Roberts et al., 2007). SARS-like viruses isolated from bats displayed major differences including a deletion in the ACE2 RBM compared to human SARS-CoV (Drexler et al., 2010; Ren et al., 2008) and as a consequence were unable of using human ACE2 as an entry receptor (Becker et al., 2008). However, recently a novel SARS-like BtCoV was identified, which could use ACE2 of *Rhinolophus* bats, palm civets as well as of humans as a functional receptor (Ge et al., 2013). These findings not only provide further evidence that bats are indeed the natural reservoir for SARS-like CoVs, but also that these bat coronaviruses can directly include human ACE2 in their receptor repertoire. The detection of sequences of SARS-CoV-like viruses in palm civets and raccoon dogs (Guan et al., 2003; Tu et al., 2004) therefore probably reflects the unusually wide host range of these viruses. A similar promiscuous receptor usage is also observed for MERS-CoV which binds to DPP4 of many species (Barlan

et al., 2014; Eckerle et al., 2014; Falzarano et al., 2014; Müller et al., 2012; van Doremalen et al., 2014) as indicated earlier.

Just as SARS like and MERS-CoVs are able to use entry receptors of different host species, also several α-CoVs display promiscuity to orthologous receptors. For example, the feline APN molecule can be used as a receptor by feline (serotype II FIPV), canine (CCoV), porcine (TGEV), and human (HCoV-229E) α-CoVs in cell culture (Tresnan and Holmes, 1998; Tresnan et al., 1996). Conversely, serotype II FIPV can only enter cells expressing feline APN (Tresnan and Holmes, 1998). The ability of TGEV and CCoV to use feline APN as a receptor probably results from strong conservation of the viral-binding motif (VBM) among APN orthologs in combination with the RBDs recognizing APN in a similar fashion (Reguera et al., 2012). Though recruiting the same receptor, HCoV-229E binds another domain within APN, which apparently is also conserved in feline APN (Kolb et al., 1997; Tusell et al., 2007). Conservation of the VBM obviates the need for large adaptations within the RBD of these viruses to orthologous receptors allowing more facile cross-species transmission.

Other mutations in the S_1 CTR associated with altered tropism have been described for the β-CoV MHV. Similar to the humanized CEACAM1a-recognizing MHV variant, serial passaging of virus-infected cells resulted in the selection of viruses with an extended host range, which were subsequently shown to be able to enter cells in a heparan sulfate-dependent and CEACAM1a-independent manner (de Haan et al., 2005; Schickli et al., 1997). Two sets of mutations in the S protein were shown to be critically required for this phenotype, both of which resulted in the occurrence of multibasic heparan sulfate-binding sites. While one heparan sulfate-binding site was located in the S_2 subunit immediately upstream of the fusion peptide, the other was located in the S_1 CTR. The presence of this latter, but not of the former, domain resulted in MHV that depended on both heparan sulfate and CEACAM1a for entry. Additional introduction of the second heparan sulfate-binding site enabled the virus to become mCEACAM1a independent (de Haan et al., 2006). In addition, a mutation of the HVR of S_1 may affect CoV tropism as was demonstrated for the MHV strain JHM (MHV-JHM). The spike protein of MHV-JHM may induce receptor-independent fusion (Gallagher et al., 1992, 1993). However, deletion of residues in HVR of MHV-JHM resulted in the spike protein being entirely dependent on CEACAM1a binding for fusion (Dalziel et al., 1986; Gallagher and Buchmeier, 2001; Phillips and Weiss, 2001).

5.2 Changes in Proteolytic Cleavage Site and Other S_2 Mutations Associated with Altered Tropism

5.2.1 Changes in Proteolytic Cleavage Sites

Although the S_2 subunit does not appear to contain any RBDs, several mutations in this subunit have been associated with changes in the virus' tropism. Some of these changes affect the cleavage sites in the S protein that are located at the S_1/S_2 boundary or immediately upstream of the fusion peptide (S_2' cleavage site). As these cleavages appear to be essential for virus-cell fusion, the availability of host proteases to process the S protein is of critical importance for the virus' tropism. The importance of S protein cleavage at the S_1/S_2 boundary for the tropism of the virus is exemplified by the BtCoV HKU4, which is closely related to the MERS-CoV. Although domain B of the HKU4 S protein can interact with both bat and human DPP4, it is only in the context of bat cells, but not human cells, that the virus can utilize these molecules as entry receptors (Yang et al., 2014). In contrast, MERS-CoV can enter cells of human and bat origin via both DPP4 orthologues. This difference results from host restriction factors at the level of proteolytic cleavage activation. Two-amino acid substitutions (S746R and N762A) in the S_1/S_2 boundary of the S protein were shown to be crucial for the adaptation of bat MERS-like CoV to the proteolytic environment of the human cells (Yang et al., 2015).

Although probably not directly responsible for the tropism change associated with the enterically replicating FECV evolving into the systemically replicating FIPV, loss of a furin cleavage site at S_1/S_2 junction is observed in the majority of the FIPVs, whereas this furin cleavage site is strictly conserved in the parental FECV strains (Licitra et al., 2013). Apparently, conservation of this furin cleavage site is not required for efficient systemic replication. However, as FIPV is generally not found in the feces of cats, it may well be that loss of the furin cleavage site at S_1/S_2—as well as mutations in other parts of the genome, such as the accessory genes—may prevent efficient replication of FIPV in the enteric tracts.

Besides the influence of the S_1/S_2 cleavage site, virus tropism may also depend on the S_2' cleavage site upstream of FP1. In contrast to wild-type MHV strain A59, a recombinant MHV carrying a furin cleavage site at this position was shown to no longer depend on lysosomal proteases for efficient entry to occur (Burkard et al., 2014). As a consequence, this virus was able to infect cells in which trafficking to lysosomes was inhibited. Cleavage at the S_2' site may also be important for the tropism of PEDV, which causes major damage to the biofood industry in Asia and the Americas (Lee, 2015;

Song et al., 2015). PEDV replication in cell culture is strictly dependent on trypsin-like proteases, a requirement which is expected to limit its tropism in vivo to the enteric tract. The trypsin dependency of PEDV entry was shown, however, to be lifted after introduction of a furin cleavage site at the S_2' cleavage site by a single-amino acid substitution. Such mutations may potentially affect the spread of this virus in the pig by allowing it to replicate in nonenteric tissues in the absence of trypsin-like proteases (Li et al., 2015).

5.2.2 Other S_2 Mutations Associated with Altered Tropism

Mutations in other parts of the S_2 subunit than those affecting the proteolytic cleavage sites may also influence the tropism of different CoVs. Several studies report a correlation between mutations in the HR1 region of FCoVs and the conversion of FECV into FIPV (Bank-Wolf et al., 2014; Desmarets et al., 2016; Lewis et al., 2015). Such a correlation appeared even more convincing for mutations found in the recently identified FP2 (Chang et al., 2012; Ou et al., 2016). While these correlations suggest an important role for the S protein in the transition of FECV into FIPV, the causal relationship between these mutations in S and FIP remains to be determined. It is plausible, however, that such mutations may play a role in the acquired ability of FIPVs to infect macrophages. Indeed, for serotype II FCoV, the ability to replicate in macrophages was shown to be determined by residues located in the C-terminal part of the S_2 subunit, although the responsible residues were not identified (Rottier et al., 2005).

Also for other CoVs, mutations in the S_2 subunit have been linked to changes in the virus' tropism. A serially passaged MHV-A59 virus was shown to obtain mutations (M936V, P939L, F948L, and S949I) in and adjacent to the HR1 region which conveyed host range expansion of the mutant virus to normally nonpermissive mammalian cell types in vitro (Baric et al., 1999; McRoy and Baric, 2008). Contrary, Krueger et al. reported three mutations in the S_2 subunit of MHV-JHM (V870A located upstream of the S_2' cleavage site and A994V and A1046V located in the HR1 region) all of which reduced the CEACAM1a-independent fusogenicity of this virus (Krueger et al., 2001). Many studies on MHV-JHM point to a crucial role of a leucine at amino acid position 1114 in S protein fusogenicity. The MHV S cryo-EM structure demonstrates that the L1114 residue is located in the central helix and contributes to interprotomer interactions. A L1114F substitution in the MHV-JHM S protein

was observed in a mutant strain of JHM and correlated with an increased S_1–S_2 stability and the loss of the ability to induce CEACAM1a-independent fusion (Taguchi and Matsuyama, 2002), while a substitution of the same residue to an Arg (L1114R) reduced the neurotropism of this virus (Tsai et al., 2003). Mutants resistant to a monoclonal antibody (Wang et al., 1992) and soluble receptor (Saeki et al., 1997) also correlate with substitutions at this specific residue, illustrating the importance of this residue in S fusogenicity. For the MERS-CoV, mutations in HR1 have been identified that are thought to be associated with its adaptive evolution (Forni et al., 2015). Among these sites, position 1060 is particularly interesting, as it appears to correspond to substitutions found in MHV and IBV that modify the tropism of these viruses (MHV: E1035D; IBV: L857F; Navas-Martin et al., 2005; Yamada et al., 2009). Substitution E1035D in HR1 of MHV was shown to restore the hepatotropism of an otherwise non-hepatotropic MHV, the latter resulting from mutations in the S_1 NTR and the S_1/S_2 cleavage site. These studies collectively indicate that mutations in and close to the HR regions may affect CoV tropism, possibly by affecting the metastability and consequently fusogenicity of the S protein and/or the formation of the postfusion six-helix bundle.

6. CONCLUDING REMARKS

It appears that changes in the S protein associated with altered tropism can be found in several regions of the spike protein. These regions obviously include the NTR and CTR of S_1 that are involved in the interaction with attachment and/or entry receptors. Substitutions within the S_1 RBDs may convey an altered viral tropism by adaptation of the virus to new or orthologous entry receptors. In addition, the S protein cleavage sites are important for host tropism as the processing of these sites by host proteases will critically affect the removal of the S_1-mediated locking of the S_2 prefusion conformation by shedding of S_1 (S_1/S_2 cleavage site) and the release of the fusion peptide(s) (S_2' cleavage site). Finally, changes in S_2 (particularly in the HR regions) may compensate for yet suboptimal spike binding to orthologous receptors by which low relative affinity interactions suffice to induce the required conformational changes of the S protein that ultimately result in the formation of the postfusion six-helix bundle and virus-cell fusion.

The observation that the different domains of the S protein all contribute to the tropism of CoVs is indicative of a coordinated interplay between these

domains. This interplay has also been inferred from several studies, which reported changes in one S protein subunit often to be accompanied by adaptations in the other subunit (Saeki et al., 1997; Wang et al., 1992). In addition, the interplay between S_1 and S_2 has also been shown to be important for changes in the tropism of the virus as indicated earlier (de Haan et al., 2006; Navas-Martin et al., 2005). The recently published cryo-EM structures of CoV spike proteins (Kirchdoerfer et al., 2016; Walls et al., 2016) now provide structural evidence for the complex interplay between the subunits and domains of the S protein.

From all these studies, a picture arises in which the S protein is progressively destabilized through receptor engagement and proteolytic activation. In this process the S_1 subunits serve as a safety pin that stabilizes the fusogenic S_2 trimer. The safety pin is discharged upon interaction with a specific receptor and processing by host cell proteases and thereby gives way to conformational changes of the instable S_2 subunit. Subsequent release of the fusion peptide may resemble the pulling of the trigger which inevitably results in fusion of viral and host membranes through interaction of the heptad repeats regions.

Based on the presented data we propose a model in which the ability of a CoV to cross the host species barrier is critically dependent on the interplay between the different regions of the S proteins. In this model, the probable low affinity of the S_1 RBD for a novel receptor must be compensated by sufficiently low S_2 metastability, which depends on both proteolytic cleavage of the S protein and the S_2 interprotomer interactions. These required S protein characteristics may be generated during naturally occurring quasispecies variation and may result in the ability of the virus to replicate in and adapt to a new host.

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