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Microreview

Hosting the severe acute respiratory syndrome coronavirus: specific cell factors required for infection

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Summary

As with all viruses, the severe acute respiratory syndrome coronavirus (SARS-CoV) utilizes specific host cell factors during its infection cycle. Some of these factors have been identified and are now increasingly scrutinized as targets to intervene with infection. In this brief review, we describe the current understanding of how the SARS-CoV is able to use the cellular machinery for its replication.

Introduction

Viruses are infectious agents incapable of reproduction on their own. They replicate within cells by diverting the cellular machinery to their own advantage. Because these host–pathogen interactions are ultimately the basis of disease, knowledge about this interplay is of great interest, not the least for the development of rational strategies to combat infections. In general, much is known about the pathogens but little about the contributions of the host. In this short review we focus on the severe acute respiratory syndrome coronavirus (SARS-CoV) describing what is currently known of the cell's contributions during the successive phases of the infection cycle, i.e. entry, replication and assembly (Fig. 1). Where it is necessary and possible, reference is made to relevant information about these processes for other coronaviruses.

Coronaviruses constitute a family of enveloped, positive-stranded RNA viruses that usually cause respiratory or intestinal infections in various species. Their relevance has increased considerably by the recent emergence of new human coronaviruses (HCoV) such as the SARS-CoV (Drosten *et al*., 2003; Ksiazek *et al*., 2003; Peiris *et al*., 2003), HCoV-NL63 (van der Hoek *et al*., 2004) and HCoV-HKU1 (Woo *et al*., 2005), which all cause severe respiratory tract disease. Coronaviruses can be divided into three groups on the basis of serological and genetic criteria. The taxonomic position of the SARS-CoV has not been formally assigned, but the virus was considered most closely related to the coronaviruses of group 2 (Snijder *et al*., 2003), which includes among others the mouse hepatitis virus (MHV). Coronavirus particles contain a helical nucleocapsid structure, consisting of one copy of the viral genomic RNA packaged by multiple copies of the nucleocapsid (N) protein. Their lipid envelope accommodates three to four membrane proteins, with the membrane (M), envelope (E) and spike (S) proteins being invariably present (reviewed by de Haan and Rottier, 2005). The SARS-CoV contains a 29.7 kb genomic RNA (Marra *et al*., 2003; Rota *et al*., 2003) with the typical coronavirus genome organization, which is characterized by the occurrence of a distinctive set of genes positioned in a fixed order: 5′-1a-1b-S-E-M-N-3′. The two partly overlapping open reading frames (ORFs) 1a and 1b comprise approximately two-thirds of the genome and encode all viral components required for viral RNA synthesis. In addition, the SARS-CoV contains a set of accessory genes of unclear function between the S and E and the M and N genes (Yount *et al*., 2005), which differ distinctly from the so-called group-specific genes found in other coronaviruses.

Entry

Coronavirus entry is driven by the S glycoprotein, which is a class I fusion protein (Bosch *et al*., 2003). Trimers of the S protein form the peplomers that radiate from the virion envelope, giving it its characteristic corona solis-like appearance. While the S proteins of some coronaviruses – notably those of group 2 – are cleaved during their maturation by furin-like enzymes (de Haan *et al*., 2004), this does not appear to be the case for the SARS-CoV S protein (Xiao *et al*., 2003; Bisht *et al*., 2004; Song *et al*., 2004; Simmons *et al*., 2005). Nevertheless, an amino-terminal S1 and a carboxy-terminal S2 subunit can be distinguished, which are responsible for receptor binding

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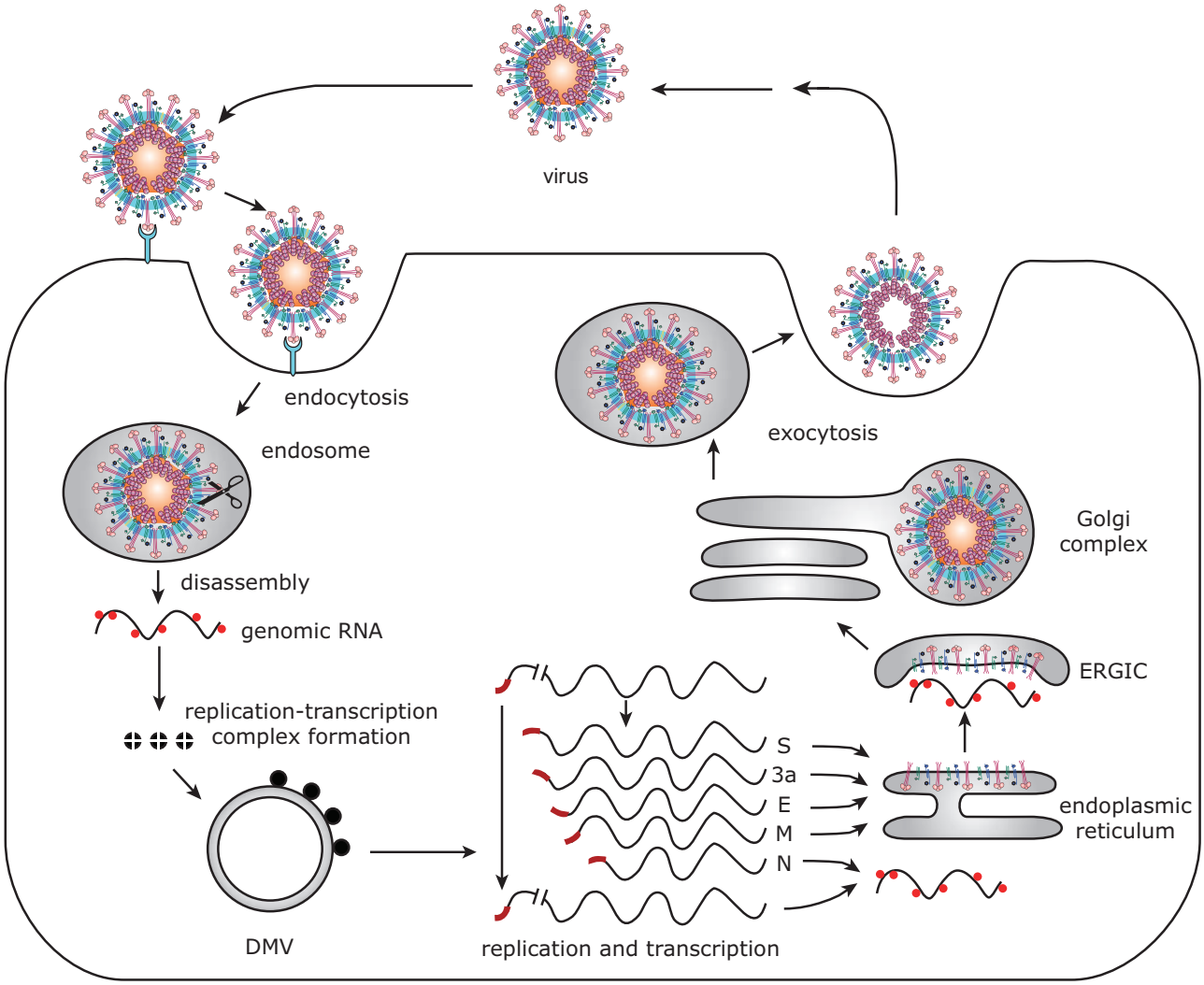

Fig. 1. SARS-CoV life cycle. The infection cycle of the SARS-CoV starts with the binding of the virion to the receptor ACE2. After endocytosis, the S protein is cleaved by cathepsin L (represented by the scissors), after which the viral envelope fuses with the host cell membrane. Next, the virus disassembles, releasing its genomic RNA into the cytoplasm of the host cell. Translation of the replicase genes produces two large precursor proteins (pp1a and pp1ab) the many cleavage products of which (nsp1-16) collectively constitute the functional replication–transcription complexes on double-membraned vesicles (DMVs). Genes located downstream of the replicase genes are expressed from a 3′-coterminal nested set of subgenomic mRNAs, each of which contains a short 5′ leader sequence derived from the 5′ end of the genome (shown in red). Many copies of N protein package the genomic RNA into a helical nucleocapsid. The envelope proteins (S, 3a, E and M) are inserted into the ER membrane, and accumulate in the ER-to-Golgi intermediate compartment (ERGIC) to meet the nucleocapsid and, subsequently, to assemble into particles by budding. Finally, the complete virions are transported out of the cell via the constitutive secretory pathway.

and membrane fusion respectively. The receptor-binding domain in the SARS-CoV S protein has been mapped to residues 318–510 (Babcock *et al*., 2004; Wong *et al*., 2004). It appears that in different coronaviruses the receptor-binding domain occurs in different regions of the S1 subunit (reviewed by de Haan and Rottier, 2005). The ectodomain of the S2 subunit contains two heptad repeat (HR) regions, a sequence motif characteristic of coiled coils, while the fusion peptide is predicted to be located immediately upstream of the first HR region (Bosch *et al*., 2004). Binding of the S1 subunit to the receptor is thought to trigger a series of conformational changes that ultimately results in the formation of an antiparallel heterotrimeric six-helix bundle by the two HR regions – characteristic of class I fusion proteins – and, consequently, the close colocation of the putative fusion peptide and the transmembrane domain. These structural rearrangements in the S protein generate the energy that drives the fusion of the viral and cellular lipid membrane, a process in which cholesterol appears to be an essential cofactor (Thorp and Gallagher, 2004).

The first host component essential for SARS-CoV infection is the angiotensin-converting enzyme 2 (ACE2). It was identified as the main receptor soon after the identi-

fication of the virus as the cause of SARS (Li *et al*., 2003; Wang *et al*., 2004). ACE2 is a metalloprotease that is abundantly expressed in human heart, kidney, testis, gastrointestinal tract and lungs (Kuhn *et al*., 2004). Using a mouse model, ACE2 was also shown to be the crucial receptor *in vivo* (Kuba *et al*., 2005). While human ACE2 functions as an efficient receptor for the 2002–03 SARS-CoV, this is much less so for the 2003–04 viruses and for the palm civet SARS-CoV (Li *et al*., 2005a). These differences are explained by amino acid substitutions in the receptor-binding domain of the SARS-CoV S protein (Li *et al*., 2005a,b; Qu *et al*., 2005). Conversely, rat ACE2, which does not support infection by SARS-CoV, and mouse ACE2, which does so only very inefficiently (Li *et al*., 2004), contain amino acid differences relative to the human sequence that are also likely to interfere with ACE2–S interaction (Li *et al*., 2005b). Enzymatic activity of ACE2 was shown not to be required for the receptor to be functional (Moore *et al*., 2004), nor does binding of SARS-CoV S protein to ACE2 alter its enzymatic activity (Kuhn *et al*., 2004). S protein binding does, however, result in downregulation of ACE2 cell surface expression (Kuba *et al*., 2005). Strikingly, ACE2 was shown to protect, in a mouse model, against lung damage caused by SARS-CoV and other agents. Therefore, downregulation of ACE2 may contribute to the severity of lung pathology observed upon SARS-CoV infection (Imai *et al*., 2005).

Unlike most other group 2 coronaviruses, SARS-CoV does not have a heamagglutinin-esterase-like attachment factor. Yet, several lectins (DC-SIGN, DC-SIGNR/L-SIGN/CD209L and LSECtin) have been demonstrated to function as such by binding to the S protein (Jeffers *et al*., 2004; Marzi *et al*., 2004; Yang *et al*., 2004; Gramberg *et al*., 2005; Chan *et al*., 2006). Expression of these lectins in the absence of ACE2 did not lead to infection driven by retroviral particles containing the SARS-CoV S protein; in the presence of ACE2 infectious retroviral entry was augmented (Marzi *et al*., 2004; Gramberg *et al*., 2005). Furthermore, it was found that the presence of L-SIGN allowed very inefficient entry of SARS-CoV (Jeffers *et al*., 2004), although in another study this lectin did not appear to facilitate infection of SARS-CoV in ACE2 expressing cells (Chan *et al*., 2006). Nevertheless, DC-SIGN and L-SIGN expressing cells, as well as dendritic cells, which could not be infected themselves, were able to promote cell-mediated transfer of virus to susceptible target cells (Marzi *et al*., 2004; Yang *et al*., 2004; Chan *et al*., 2006). Interestingly, L-SIGN also appears to play a protective role in SARS-CoV infection. Compared with cells heterozygous for L-SIGN, cells homozygous for L-SIGN (with respect to a polymorphism in the extracellular neck region) show a higher binding capacity for SARS-CoV, higher proteasome-dependent viral degradation and a lower capacity for cell-mediated transfer of virus (Chan *et al*., 2006). These results correlate with a genetic risk association study in which individuals homozygous for L-SIGN appear less susceptible to SARS-CoV infection (Chan *et al*., 2006).

The entry pathway of coronaviruses has not been clearly established. While some coronaviruses appear to use an endosomal route of infection (Nash and Buchmeier, 1997; Hansen *et al*., 1998; Nomura *et al*., 2004), others seem to enter cells at the plasma membrane (Nash and Buchmeier, 1997). Conceivably, the route of entry taken is determined both by the coronavirus (strain) and by the host cell. In the case of SARS-CoV, lysomotropic agents were demonstrated to block the entry both of lentiviral particles pseudotyped with the SARS-CoV S protein (Simmons *et al*., 2004; Yang *et al*., 2004; Huang *et al*., 2006) and of SARS-CoV itself (Huang *et al*., 2006), indicating that acidification of endosomes is required for entry. However, activation of the S protein by proteases was sufficient for bypassing the entry inhibition caused by lysomotropic agents (Matsuyama *et al*., 2005; Simmons *et al*., 2005) and resulted in cell–cell fusion (Simmons *et al*., 2004; Matsuyama *et al*., 2005). Apparently, low pH *per se* is not required for fusion. Accordingly, infection mediated by the SARS-CoV S protein could be inhibited by specific inhibitors of the pH-sensitive endosomal protease cathepsin L (Simmons *et al*., 2005; Huang *et al*., 2006). Because the SARS-CoV S protein is not cleaved during biogenesis and maturation of the virion, it is feasible that cathepsin L cleaves the protein closely upstream of the SARS-CoV fusion peptide thereby locating this hydrophobic peptide near to the newly generated N terminus of the membrane-anchored subunit, as is generally the case for class I fusion proteins. Whether cathepsin L (-like) cleavage is essential for the activation of S proteins of coronaviruses in general or, for instance, just for those not processed by furin enzymes has yet to be established. Interestingly, the infection by HCoV-NL63, which also uses ACE2 as its receptor (Hofmann *et al*., 2005) and whose S protein is uncleaved, is less low-pH sensitive and is not blocked by cathepsin L-inhibitors (Huang *et al*., 2006), indicating that two coronaviruses that utilize the same receptor can enter cells through distinct mechanisms.

RNA replication

Nothing is known about the initial fate of the viral nucleocapsid once it is delivered to the cell's cytoplasm by the fusion of viral and cellular membranes. Whether it disengages from the viral envelope components, whether it is transported to a specific location and how it is disassembled to allow translation of the viral RNA remains elusive. SARS-CoV genome expression starts with the translation of the very large ORF1a and ORF1b genes. The more downstream ORF1b is translated by translational

readthrough using a ribosomal frameshift mechanism for which a 'slippery' sequence and a pseudoknot are required (Thiel *et al*., 2003). As a result, two very large polyproteins (pp1a and pp1ab; 4382 and 7073 amino acids, respectively) are produced, which are predicted to be cleaved by two viral proteinases into 16 subunits (nsp1-16) (Snijder *et al*., 2003; Ziebuhr, 2004). These cleavage products collectively constitute the replication–transcription complex. The replication–transcription complex mediates both genome replication and the transcription of a 3′-coterminal nested set of subgenomic mRNAs from which the genes located downstream of ORF1b are expressed. Each of the subgenomic mRNAs contains a short 5′- leader sequence derived from the 5′ end of the genome, which is acquired by a mechanism that involves discontinuous synthesis of subgenomic minus strands and which is dependent on transcription regulatory sequences located upstream of most genes (reviewed by Sawicki and Sawicki, 2005).

Several cleavage products of the SARS-CoV pp1a and pp1ab (nsp3, 4 and 6) contain hydrophobic domains that presumably mediate their anchoring in cellular membranes where they may function in the recruitment of the viral replication complex (Harcourt *et al*., 2004; Ziebuhr, 2004; 2005). Although the precise mechanism that leads to the formation of these replication complexes has not yet been elucidated, increasing evidence indicates that coronaviruses attach their replication machinery to the limiting membrane of autophagosomes. In the cellular process of autophagy, compartments bound by double membranes sequester regions of the cytosol and then mature and degrade their cytosolic contents (Kirkegaard *et al*., 2004). Cells infected with either MHV or SARS-CoV accumulate large double-membraned vesicles (DMVs) to which the viral replication complexes are localized (Gosert *et al*., 2002; Goldsmith *et al*., 2004). Consistently, the coronavirus replication complexes were demonstrated to colocalize with markers of autophagosomes (Prentice *et al*., 2004a,b). Furthermore, the cellular machinery of autophagy is essential for the formation of these double-membraned compartments in cells infected with MHV and the replication of this virus is severely impaired in its absence (Prentice *et al*., 2004a). However, MHV replication complexes have also been localized to late endosomal membranes using electron microscopy (van der Meer *et al*., 1999). To what extent and how coronaviruses exploit these intracellular transport routes, remains to be elucidated.

Once the replication–transcription complexes are formed, coronaviral RNA replication and transcription not only involves viral proteins, but also several cellular proteins. In addition to the N protein, which is also required for efficient RNA replication (Almazan *et al*., 2004; Schelle *et al*., 2005), several heterologous nuclear ribonucleoprotein (hnRNP) family members (hnRNPA1, PTB and SYNCRYP), known to be involved in premRNA processing, were found to bind to different regions of the (+) and (–) strand genomic RNA and to affect coronavirus replication and transcription (reviewed by Shi and Lai, 2005). Other RNA-binding proteins have also been suggested to play a role in coronavirus replication, such as m-aconitase (Nanda and Leibowitz, 2001) and poly-A-binding protein (Spagnolo and Hogue, 2000). The N proteins from MHV and SARS-CoV were shown to interact with hnRNAP A1 (Wang and Zhang, 1999; Luo *et al*., 2005). Interactions between viral RNA, N protein and cellular proteins such as hnRNPs may result in the formation of ribonucleoprotein structures that mediate replication and/or discontinous transcription by the replication–transcription complexes. Clearly, our understanding of this elementary process of the infection cycle, particularly of the host cell contributions, is rudimentary.

Assembly

Once sufficient levels of new genomic RNA and structural components start to accumulate, assembly of virions ensues. Coronaviruses assemble their particles by budding of the helical nucleocapsid through membranes early in the secretory pathway, in particular the endoplasmic reticulum to Golgi intermediate compartment (ERGIC). The involvement of host cellular factors in this process has hardly been explored. Work has so far been concentrated on the viral components and their interplay (reviewed by de Haan and Rottier, 2005). It is clear that the M protein, a triple spanning membrane protein, is the key player in coronavirus assembly. It is the most abundant protein in the virion, which, through its interactions with every known component of the virion, orchestrates the entire assembly process. The M protein exerts its central role in assembly by selecting and organizing the viral envelope components at the assembly sites and by mediating the interactions with the nucleocapsid to allow the budding of virions. Two types of interactions appear to effect the incorporation of the nucleocapsid into the virion: protein–protein interactions between the M protein and the N protein and protein–RNA interactions between the M protein and the viral genome. At the level of the membrane the M protein interacts with itself, to generate the basic molecular framework of the envelope, with the E protein, to induce the budding and release of the M protein-modified membrane, and with the S protein, to assemble the spikes into the viral envelope. In addition, the SARS-CoV M protein interacts with the 3a protein, resulting in its incorporation into particles (Ito *et al*., 2005). The 3a protein is another triple-spanning membrane protein, hence similar to M, also in the fact that it is (O-)glycosylated. Interestingly, also for some other coronaviruses such triple-spanning proteins

have been predicted (Oostra *et al*., 2006), though their occurrence in virions has not been observed. These latter proteins have been shown to be dispensable, which also appears to be the case for 3a (Yount *et al*., 2005).

The coronavirus envelope proteins have the capacity to assemble, just by themselves, uniform envelopes, which have the same appearance and dimensions as normal virions. Such virus-like particles form independently of the N protein, E and M being the minimally required proteins (Vennema *et al*., 1996; reviewed by de Haan and Rottier, 2005). This has also been reported for the SARS-CoV (Mortola and Roy, 2004; Hsieh *et al*., 2005), although others have claimed the M and N proteins to be necessary and sufficient for formation of virus-like particles (Huang *et al*., 2004). In this respect, it is perhaps worth mentioning the varying importance of the E protein for the assembly of infectious coronaviruses. Thus, while the E protein is essential for the production of infectious porcine transmissible gastroenteritis virus (Ortego *et al*., 2002), this is not the case for MHV (Kuo and Masters, 2003) and SARS-CoV (L. Enjuanes, pers. comm.). The small hydrophobic E protein is a viroporin; it forms ion channels (Wilson *et al*., 2004) and is able to alter the membrane permeability of cells (Madan *et al*., 2005). Viroporins of other enveloped viruses were also shown to enhance the membrane permeability and to promote virus budding and release (reviewed by Gonzalez and Carrasco, 2003).

In general, viral glycoproteins determine the site of virion budding. For coronaviruses this is, however, not so apparent because the most obvious candidate, the M protein localizes to the Golgi complex, beyond the site of budding. Also the E and S proteins do not localize to the budding compartment (Nal *et al*., 2005; reviewed by de Haan and Rottier, 2005). It might well be that the lateral association of the envelope proteins creates novel localization signals that direct the multimeric complexes to the budding site. Alternatively, interactions of the M protein with the nucleocapsid could determine the localization of budding. Whereas early in infection the N protein colocalizes with the replication complexes, which are almost entirely discrete from the sites of M protein accumulation, at later times the helicase and the N proteins appear to colocalize with the M protein (Bost *et al*., 2001). The relocation of the helicase–N complex may serve as a mechanism to deliver the newly synthesized RNA and nucleocapsids to the assembly sites and to facilitate the retention of the M protein in the intermediate compartment. Considering what is becoming known about the assembly processes of other viruses, particularly of HIV-1 (Freed, 2004), it is safe to expect that also coronaviruses rely on an arsenal of host factors for their virion assembly and budding. Yet, none has so far been identified.

Besides the coronavirus proteins also the coronavirions themselves are subject to intracellular maturation processes while on their way through the constitutive secretory pathway out of the cell. Thus, while the N-glycans of the SARS-CoV M and S proteins mature (Nal *et al*., 2005) and the 3a protein becomes O-glycosylated (Oostra *et al*., 2006), the coronavirus particles undergo structural maturation during their transport through the Golgi complex (reviewed by de Haan and Rottier, 2005). The significance of this maturation process, which is seen morphologically as a rearrangement of the inner ribonucleoprotein accompanied by shrinkage of the particle, remains unclear. It has so far not been reported for SARS-CoV.

Perspective

Ever since its identification as the cause of SARS, knowledge about the SARS-CoV has accumulated at breathtaking pace, obviously by profiting from the pre-SARS insights in coronavirology. However, as for other coronaviruses, this knowledge is mostly limited to aspects of the molecular biology of the virus and of the disease process. Relatively little do we know about the structure of the virion and, as outlined in this review, about the contributions of the host cell to infection. It is only now that the latter issue is becoming amenable to research due in particular to the explosive technical developments in the fields of (functional) genomics and proteomics.

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