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Review

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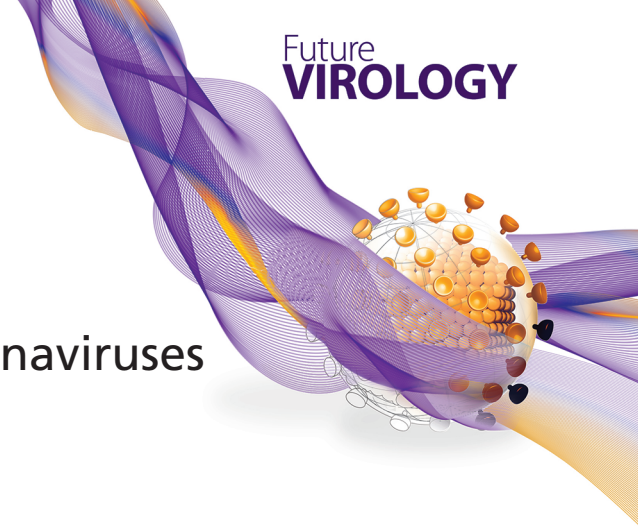

Membrane binding proteins of coronaviruses

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Coronaviruses (CoVs) infect many species causing a variety of diseases with a range of severities. Their members include zoonotic viruses with pandemic potential where therapeutic options are currently limited. Despite this diversity CoVs share some common features including the production, in infected cells, of elaborate membrane structures. Membranes represent both an obstacle and aid to CoV replication – and in consequence – virus-encoded structural and nonstructural proteins have membrane-binding properties. The structural proteins encounter cellular membranes at both entry and exit of the virus while the nonstructural proteins reorganize cellular membranes to benefit virus replication. Here, the role of each protein in membrane binding is described to provide a comprehensive picture of their role in the CoV replication cycle.

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Coronaviruses (CoVs) are enveloped positive sense RNA viruses causing a variety of diseases in man and animals and are considered to be the largest of the RNA viruses, with genomes ranging from 27–32 kb [1]. CoVs belong to the Coronaviridae family and contain two subfamilies Orthocoronavirinae and Letovirinae. Further, the Coronaviridae family is grouped into the suborder Cornidovirineae, which together with the suborders Abnidovirineae, Arnidovirineae, Mesnidovirineae, Monidovirineae, Ronidovirineae and Tornidovirineae forms the Nidovirales order [1,2], so named for the overlapping set of transcripts used by all members to encode viral proteins. The Coronaviridae are further subdivided phylogenetically into four genera, α, β, γ and δ [3]. The CoVs include members classified as emerging viruses, viruses that are able to cross the species barrier and cause pathology in a new target species. Two such recent events are the highly pathogenic severe acute respiratory syndrome-related CoV (SARS-CoV) that emerged in Southern China in 2003 and Middle East respiratory syndrome-related CoV (MERS-CoV), which appeared in Saudi Arabia in 2012 [4,5]. There is no effective treatment or licensed vaccine for either virus, which emphasizes the need to further understand CoV biology as a route to improved future intervention [6,7].

CoVs are structurally complex (Figure 1) with purified virus particles consisting of four or five structural proteins along with a variety of minor components including nonstructural and host cell-derived proteins [8]. All viruses have Nucleocapsid (N), Spike (S), Envelope (E) and Membrane (M) structural proteins and some also encode a hemagglutinin–esterase (HE) protein [1]. Despite their complexity and range of function however, [9,10] the structural proteins of CoVs occupy only about a third of the coding capacity of the genome. A much larger section of the genome, some two-thirds located at the 5′ end encode two long open reading frames 1a and 1b that together encode the nonstructural proteins of the virus. Each sequence is translated first as a polyprotein precursor, pp1a and pp1ab, the latter achieved by a frameshift event at the end of the 1a coding sequence. The polyproteins include several viral proteases that together process pp1a and pp1ab into 16 nonstructural proteins (nsp1–16), which are required at various stages of the virus replication cycle [1]. As an enveloped virus, the virus surface proteins, S, M and E encounter cellular membranes at the initiation of infection, again during the replication cycle when they are translated and incorporated into the endoplasmic reticulum and endoplasmic reticulum Golgi intermediate compartment (ERGIC) [11,12] and finally in the secretory pathway where budding of the mature virions occurs (Figure 2) [9,13]. In addition, many of the nonstructural proteins also interact with membranes as, in common with

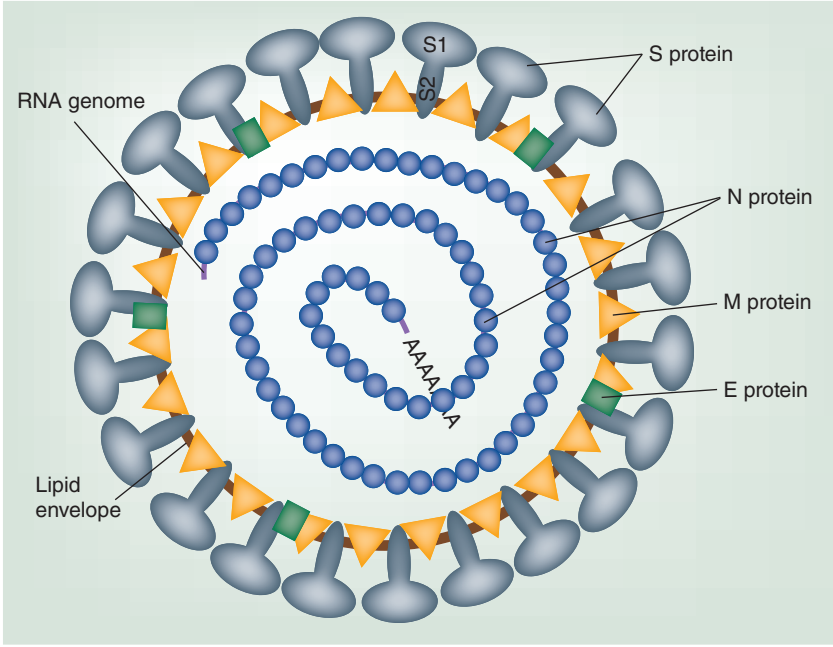

Figure 1. Schematic representation of a coronavirus particle. The structural components of the virus are indicated. Small amounts of host cell and virus nonstructural proteins, presumed to be captured nonspecifically during the budding process, are also found in virions but are not illustrated.

other positive strand RNA viruses, virus replication takes place in specialized cellular compartments induced by viral proteins which modify host membranes or organelles to set up sites for replication that are hidden from the cellular inducers of innate immunity [14]. The combination of multiple membrane interacting factors and multiple sites of membrane interaction make CoVs one of the more challenging virus-membrane interaction models available.

Structural protein interactions

The fusion process between viral and host membranes, mediated in CoVs by the S protein, is a crucial step in enveloped virus infection [15,16]. The S protein is a large class I fusion protein responsible for virus binding to target cells via cell surface receptors, which for CoVs can range from simple sugars to complex proteins (reviewed in [17,18]). For example the entry receptor for MERS-CoV infection has been identified as dipeptidyl peptidase-4 (DPP4) found on a variety of cell types including epithelial cells of the respiratory tract [19]. As a result, receptor distribution and the CoV–S-receptor interaction often defines tissue tropism and host range [18,19]. The S protein consists of two subunits, S1 and S2, with S1 at the N-terminus providing the receptor binding function and S2 at the C-terminus providing fusion activity [15]. The subunits are cleaved from the complete S by host cell proteases including members of the cathepsin family and transmembrane protease serine 2 (TMPRSS2) [20]. Following receptor binding by S1 and uptake into a vesicle the fusion mechanism of S2 acts to bring the viral and cellular membranes into such close proximity that fusion occurs [21,22]. The S2 sequence contains conserved regions that are necessary for function, notably a fusion peptide and two conserved heptad repeats (HR) [18]. Briefly, significant conformational change occurs in the late clathrin-coated endocytosed vesicle leading to release of the fusion peptide to interact with the vesicle membrane, provided that S has been cleaved into its requisite subdomains [23]. The collapse of S2, which is now bridging the virus and cellular membranes, pulls the two membranes together with HR1 and HR2 forming the canonical 6-helix bundle first described for CoVs in mouse hepatitis virus (MHV) [24].

In terms of sequence and location precise fusion peptides (FP) have yet to be defined for all CoVs [25] as recognition of the FP motif within the large spike protein can be difficult. However, bioinformatics analysis suggests that at least part of the fusion peptide is located near the N-terminus of S2 where a conserved motif with properties consistent with those expected of an FP, IEDLLF, occurs across the CoV family. This motif demonstrates very little variation and when substitutions are found, they are conservative replacements consistent with an essential function [26,27]. The motif is not located at the N-terminus of HR1 as suggested in some S protein cleavage maps (e.g., ref [21])

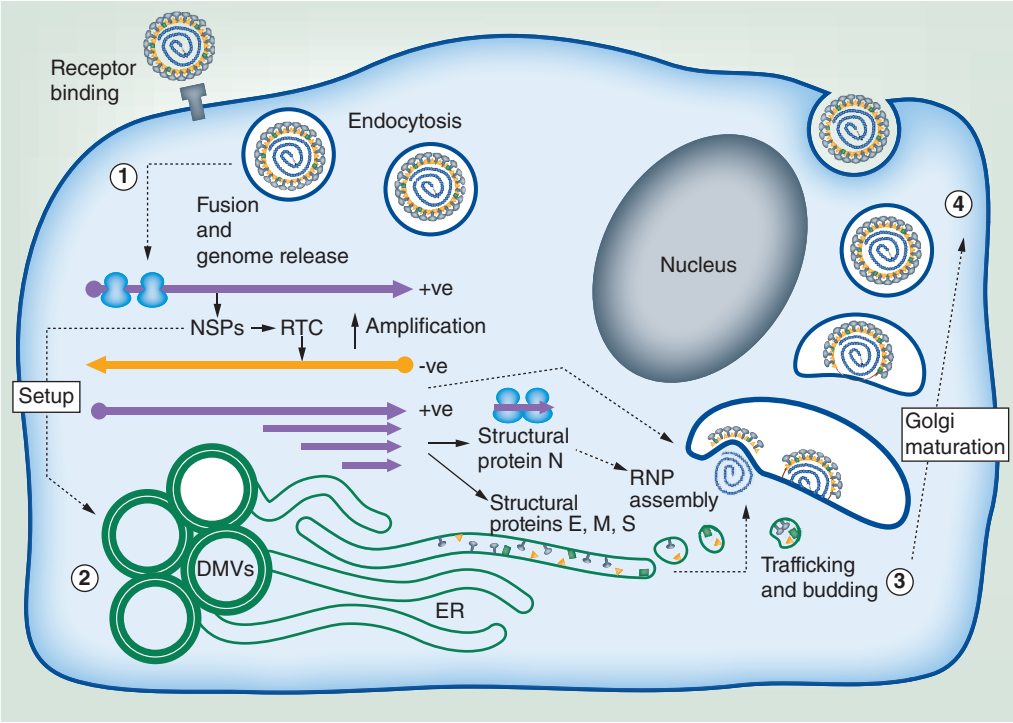

Figure 2. The coronavirus replication cycle highlighting areas where membrane interaction occurs. (1) Most Coronaviruses enter by receptor mediated endocytosis. The positive sense genomic RNA is released into the cytoplasm and translated into the initial virus polyproteins which encode the nsp. (2) The nsp stimulate the production of DMVs and establish the replication transcription complexes (RTC), which produce the -ve strand replicative intermediate from which more +ve strand genomes and mRNAs are produced. Translation of the N mRNA produces the N protein in the cytoplasm which combines with the new genomes to form RNPs while translation of the remaining structural proteins, M, E and S occurs in the ER where they accumulate in the ERGIC and cis-Golgi. (3) Virus assembly begins and completes as the protein cargos migrate through the Golgi stacks resulting in new virus particles in vesicles (4), which eventually fuse with the plasma membrane.
DMV: Double-membrane vesicle; ER: Endoplasmic reticulum; ERGIC: Endoplasmic reticulum Golgi intermediate compartment; nsp: Nonstructural protein; RTC: Replication transcription complex.

but immediately follows the second, S2′ cleavage site, originally mapped in SARS-CoV S and later in MERS CoV S [26,28,29]. A sequence which includes this motif has been shown directly for SARS-CoV to act as a fusion peptide when tested in an *in vitro* binding assay with multilamellar vesicles (MLVs) where it reorders membranes in a calcium-dependent manner [30].

The endodomain of S2 can been subdivided into two regions, a cysteine-rich region at the N-terminus and a carboxy-terminal region rich in charged residues [31–33]. It has been shown that clusters of cysteine residues are important for the palmitoylation of S. No particular cysteine residue is critical but in a study of fusion competence and replication in MHV a total of at least three cysteine residues was required [34] and other studies have confirmed that the cysteine-rich region is necessary for syncytium formation during viral infection [35,36]. While membrane binding and deformation is clearly a property of the FP sequence, propelled into the membrane by the conformational changes in S, palmitoylation of S may serve to stabilize the protein during its interactions with lipid rafts in the target membranes to allow time for fusion to occur. Recently, several CoV-S proteins including HCoV-HKU1, MHV, HCoV-NL63, SARS-CoV and MERS-CoV have had their structures solved at atomic resolution following imaging using cryo-electron microscopy [37–40]. All the confirmed structures of S are in their prefusion state and most have had their cleavage sites mutated to enhance S stability in order to enable the imaging process. As a result there is limited knowledge of the CoV FP within the fusion active conformation or of its structural characteristics when interacting with lipid bilayers after proteolytic processing at the S2` site [30,38,40].

By contrast with S, the CoV envelope protein (E) is a small hydrophobic integral membrane protein ranging from 76 to 109 amino acids. It has an N-terminal domain, a long α-helical transmembrane domain and a C-terminal hydrophilic domain and is found as a minor component in all CoV groups [41,42]. The E protein is also palmitoylated

at all three of its Cys residues [43] but the role of this secondary modification is debated. For MHV-CoV, single Cys residue changes do not significantly impair virus growth but modification of all three residues results in severe attenuation [44,45]. For SARS-CoV; however, triple mutation of the conserved Cys' does not impact secretion of virus antigen from expressing cells suggesting no particular dependence on palmitoylation [46]. Two membrane topologies have been demonstrated for E protein, hairpin or transmembrane, and it has been suggested that the level of palmitoylation may moderate their relative proportion, in turn allowing modified membrane curvature [45,47]. The E protein has demonstrated functions in virus assembly and release (below) and it appears to induce membrane curvature in the ERGIC leading to membrane scission of the budding virus particle and its release [48]. Envelope protein also interacts with the M protein and mutants of M that are unable to bud from cells can be complemented by mutated forms of E [49,50]. The membrane curving properties of E are such that co-expression of M and E is adequate for the efficient formation of virus-like particles [48,51], which can also incorporate S if it is co-expressed [52]. For many CoVs, including MHV, E protein has also been shown to have a role as an ion channel, a viroporin [53,54]. E function as a viroporin, including the trafficking of virions in the secretory pathways and membrane permeability, is essential for virus growth [55]. E also interacts with host cellular proteins including Proteins Associated with Lin Seven 1 (PALS1), which is known to maintain the epithelial cell junction, with clear implications for the virus assembly site in the Golgi [56,57]. While E function is critical for virus assembly, its viroporin activity in mobilizing calcium ions and its interactions with host tight junction cell proteins has been also implicated as a mediator of pathology in some CoV infections [55,57].

The CoV membrane protein (M) is a type III transmembrane glycoprotein and is the most abundant glycoprotein in the CoV particle. Despite variability in the primary M protein sequence the predicted secondary structures of M proteins are maintained [58]. The M protein is approximately 230 amino acids in length and is composed of three parts: a short N-terminal domain situated outside the virion membrane, three transmembrane domains and a carboxy-terminal domain situated inside the particle [59,60]. An amphipathic region situated at the end of the third transmembrane domain is well conserved in almost all Coronaviridae members [58]. CoV M proteins are characterized by N-linked glycosylation in the α and δ CoVs and O-linked glycosylation in the β CoVs [61,62] and study of chimeric M proteins has shown that the type of glycosylation is not critical for virus assembly or growth at 37°C [50]. It seems more likely that, as for many virus glycoproteins, glycosylation has a more general significance in maintaining bioactive conformation and antigenic character [63,64]. M is located among the S proteins in the virus envelope along with small amounts of E and is the primary driver of the virus budding process [51]. During assembly of the authentic virion M interacts with itself, with the nucleocapsid protein N, with E and with the S protein [44,58,65]. M protein is present as a dimer in the virion and high resolution imaging has suggested that it presents as two conformations, long and compact (M_{LONG} and $M_{COMPACT}$), which together induce membrane curvature as well as binding to the nucleocapsid [66,67].

Nonstructural protein interactions

CoV nsp 3, 4 and 6 (Figure 3) have fundamental functions in the rearrangement of host cell membranes that are required for the establishment of viral replication–transcription complexes (RTCs), also called replication organelles (RO) [68]. Indeed expression of just these proteins will induce the formation of the double-membrane vesicles (DMVs) and other structures that are characteristic of CoV-infected cells [69]. Replication complexes, intimately bound up with convoluted membrane structures, are a feature of all positive strand RNA viruses and serve at least three functions, probably connected. First, they serve to concentrate viral proteins in a microenvironment where all necessary replication factors are closely associated with the genomic RNA. Second, they exclude host factors so that the competition for resources can be focused on the virus and third they act to separate, as far as possible, the intermediates of replication, which are necessarily double stranded RNA molecules, from the host innate sensors such as TLR7 and MDA-5 [70,71].

Nsp3 has two transmembrane regions and approximately 10–16 identifiable domains (depending on the virus) within the approximately 200 kDa predicted primary translation product, eight of which are conserved [72]. It is co-translationally inserted into the endoplasmic reticulum resulting in the majority of the domains being tethered to the cytosolic side of the membrane (Figure 3). Nsp3 function is integral to CoV replication and its domains include many predicted or demonstrated to act as accessories in RNA replication such ssRNA binding and unwinding domains, as well as those for which no distinct function has yet been determined [72].

The 44 kDa CoV nsp4 protein is also a transmembrane protein, with four transmembrane helices and an internal C-terminal domain (Figure 3) and with nsp3 is an indispensable component required to produce DMVs [73,74]. All

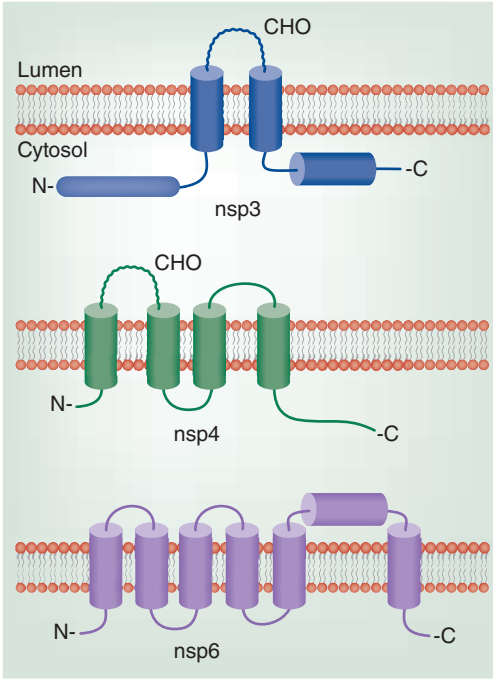

Figure 3. The suggested topology for the coronavirus nonstructural proteins associated with membrane deformation. Three proteins, nsp 3, 4 and 6 are implicated in membrane curvature and the formation of subcellular membrane organelles. The topologies suggested within the lipid bilayer (red) are supported by experimental evidence but may not be the same for all Coronaviruses. In all cases the white cylinders represent hydrophobic stretches of amino acids that are consistent with transmembrane domains although all may not be used as such (see nsp6). Only the region of the protein that interacts with the membrane is shown. N- the amino terminus of the protein, C- the carboxyl terminus of the protein, CHO- the addition of carbohydrate. The cartoon is not to scale.
Nsp: Nonstructural protein.

CoV-nsp4 molecules encode at least one predicted glycosylation site and in the case of MHV, it has been shown that mutation of the glycosylation site results in loss of virus fitness suggesting that nsp4 glycosylation is necessary for virus replication or the organization of the DMVs [75]. In an electron micrographic study, transfection of SARS-nsp3 and nsp4 alone caused considerable membrane deformation, producing a perinuclear double-walled maze-like body (MLB) [76] and the nsp3–nsp4 interaction was shown to be absolutely necessary for such membrane rearrangement. However the interaction of these two nsps was insufficient in itself to trigger membrane rearrangement and host factors such as EDEM1 and OS9 of the ER-associated degradation system have been shown to be co-factors [77,78]. Despite them being a universal feature of CoVs the size and number of DMVs does not appear to correlate directly with viral fitness, at least when virus is grown at reduced temperatures [79] nor are they a determinant of pathogenicity [80]. For the γ CoV Infectious Bronchitis Virus (IBV), nsp4 was essential and sufficient to induce membrane pairing, recognized as extensive areas of membrane accumulation or small regions of paired membrane, but expression of nsp3, nsp4 and nsp6 was required for DMV production which, even then, was poor for strain BeauR and not seen at all for strain M41. DMVs formed by IBV nsp3, nsp4, and nsp6 alone were poorly efficient when compared with DMVs formed by *Betacoronavirus* infection so supplementation of nsp4 with nsp6 is not sufficient for authentic IBV DMV production [81].

CoV nsp6 is a membrane protein of approximately 34 kDa predicted molecular mass with six transmembrane helices (Figure 3) including, in almost all viruses, a highly conserved C-terminus [82]. Although nsp6-stimulated internal cellular membrane rearrangement is observed with the addition of nsp3 and 4, nsp6 also causes membrane proliferation alone, including the formation of Atg5 and LC3II-positive vesicles classically observed in autophagy [83]. The autophagosomes produced are somewhat different from those induced by starvation; however, as although their number is higher and their size is reduced [84]. As noted above, along with nsp3 and 4, nsp6 functions to produce the canonical DMVs as well as many other types of intracellular vesicles observed in CoV infected cells such as convoluted membranes, vesicle packages, tubular bodies, large virion-containing vacuoles (LVCVs), cubic membrane structures (CMSs) and zippered ER spherules in the case of IBV [85,86]. An attenuated form of an IBV vaccine includes mutations in an nsp6 TM domain, confirming its role in virulence and replication [87].

Recruitment & modification of membranes by CoVs

As noted above, the membranous vesicles or organelles of different morphologies induced by CoVs act as a platform for the formation of replication–transcription complexes (RTCs) and sequester newly formed RNAs away from host immune sensors [88,89]. Both viral and hijacked host proteins are used in this process, taking advantage of cellular pathways and lipid modifying enzymes to the benefit of the virus [90,91]. This usurping comes about through the

commandeering of normal secretory pathways used by noninfected cells to transport and deliver protein cargos; rather than encode proteins to build DMVs anew, CoVs redirect and reorganize the cellular processes already in place [92].

Two principle mechanisms have been described for moving and delivering cargo proteins through the secretory pathway; cisternal maturation and the formation of megavesicles [93]. In both cases the detail remains incomplete [94]. During CoV infection, such as for MHV, virions have been observed in large vesicle depots resembling megavesicles derived from Golgi/ERGIC membranes, indicating that remodeling of the Golgi complex may be crucial for virion trafficking [14]. As noted, nsp6 may initiate cellular autophagy and a general ER stress response also occurs during the formation of DMVs [95,96]. Atg5 is necessary for the formation the crescent membranes and if is knocked out the yield of MHV is reduced although this is not a universal finding [97]. Although the precise mechanisms are ill defined, biological bilayers of proteins and lipids [98] are key to the separation and control of biological processes and their occurrence and composition is dynamic [99]. Bending, that is positive or negative membrane curvature, is driven by the acquisition and loss of peripheral membrane proteins, integral membrane proteins and by lipid composition [100,101]. Membrane wrapping may occur around intrinsically curved proteins in which positively charged amino acids interact with negatively charged lipid head groups, for example, in the dynamin and BAR domain interactions, also known as scaffolding [102,103]. Alternatively, crowding mechanisms may effect membrane curvature as a result of the asymmetric distribution of proteins either side of a cellular membrane [99,104], and the insertion of an amphipathic helix which can act as a wedge to expand one side of the membrane more than the other can also cause curvature as revealed by studies on influenza virus M2 protein, Epsins and Sar 1p [102,105,106].

Virus egress

During assembly, all enveloped viruses face the challenge of combining capsids proteins and genome produced in the cytosol with glycoproteins that predominantly occur in another cellular compartment, the luminal side of the ER. A cell membrane separates these components and must be breached or used in the assembly of the complete virion and this is achieved in three stages (Figure 2). First, the virus proteins coalesce on the membrane, capsid proteins grouping together underneath the patch of membrane where viral glycoproteins are embedded. Second, the membrane bulges outward to form a bud decorated by the viral transmembrane proteins and enclosing the capsid proteins and genome. Third and finally, the bud splits from the rest of the membrane by scission, a pinching-off at the base which releases the virion either into an intracellular vesicle as in the case for CoVs or directly out of the cell [1]. For many enveloped viruses these processes are actioned by viral protein interaction with host proteins of the endosomal sorting complexes required for transport (ESCRT) machinery [107]. Surprisingly however, perhaps because of incompatibility with the extensive membrane rearrangements induced in infected cells, CoVs appear not to use ESCRT proteins for egress, rather the S protein has a signal for ERGIC retention in its cytoplasmic tail [108] while the M protein locates to the ERGIC and cis-Golgi via its first TM domain where it also oligomerises [109] to drive the budding process. M–N interactions ensure that the viral RNPs also occur at these budding sites allowing the budding virus to incorporate a copy of the new genome [46,110]. The E protein, as a viroporin, has been implicated in membrane scission as E is present in virus particles at only a very low level and most is left associated with the ERGIC and *cis* Golgi consistent with a predominant role as a mediator of virus assembly and release at this location [111]. The lipid content at these locations may also enhance virus budding [112,113].

CoV membranes as antiviral targets

As CoVs cause such extensive membrane perturbation and as there is an acknowledged lack of available antiviral compounds to combat CoV-induced disease, it is not surprising that membrane rearrangement has been considered as a target for the development of inhibitors that could act as antivirals, along with the more classical targets of the polymerase and proteases [114]. Peptide therapeutics are promising antagonists in this regard as they compete directly for membrane binding or inhibit the conformational mechanisms involved and several peptides have been demonstrated to target various steps in the CoV replication cycle. A HR2 competitive peptide blocked the fusion mechanism of MERS-CoV and prevented virus entry when measured using a pseudotype assay [115] and a more complex 5 helix bundle, designed as a mimic of the final S fusion intermediate, was also active when measured similarly [116]. SARS-CoV has been inhibited similarly [117]. As membrane microdomains are implicated in CoV membrane interaction, drugs that alter microdomain composition, particularly the level of cholesterol present, have been shown to have an effect on some CoVs [118,119]. More general still is the use of drugs which alter intracellular vesicle pH and so inhibit the entry or exit of enveloped viruses, including CoVs [114,120]. Vaccines

and passive immunotherapy options have also targeted crucial CoV–membrane interactions. The predominant antibody response to S is to the S1 domain which has been shown to be a successful vaccine candidate [121,122] but the binding of antibodies directed here is subject to antigenic drift and may not be effective for all serotypes. The S2 domain by contrast is generally immunologically silent. Rare antibodies that do target S2 in the stem of S and inhibiting the fusion mechanism, are broadly reactive and so relatively impervious to serotype change [123]. The use of such broadly reactive monoclonal antibodies as therapies may be particularly suitable for the treatment of serious but sporadic CoV infections where general vaccination of the target population is not warranted or is impractical.

Future perspective

With their large, adaptable genomes and their extensive distribution in the biosphere, CoVs will certainly feature in future zoonotic outbreaks; SARS and MERS will not be the last. While vaccination remains the cornerstone of control for viral diseases, it is not quick, a new vaccine may take 15 years to develop and it is very virus specific, a MERS vaccine will not protect against SARS and vice versa. Similarly, antiviral drugs targeting the main enzyme functions of the virus risk being ineffective as a result of sequence variation in the target genes. Targeting the common physiological features of CoV replication; however, offers the possibility of developing panCoV treatments that focus on what is common to this family of viruses rather that what is distinct. There are obvious problems, viral stages that are so closely associated with host biology that toxicity would be expected, but there is also sufficient novelty, nsp-based membrane remodeling, for example, that clear targets for intervention exist. Such a strategy could offer the possibility for the development of panCoV agents of the future. More immediately, as membrane remodeling by CoVs is fundamental to immune evasion, targeting the proteins responsible for the remodeling could reveal the infection to the host immune system much sooner than would otherwise be the case and lead to the curtailment of the infection at a much earlier time, before extensive collateral damage is done. Together, a further understanding of the role of virus proteins in membrane interaction and remodeling, directly and via interaction with host factors, is likely to increase the underpinning data that lead to an increase in the therapeutic options for the control of CoV infections in the future.

Executive summary

- Coronaviruses (CoVs) are diverse, complex, adaptable viruses that have a significant impact on human health and animal productivity. Despite their diversity, common features exist, including the formation of membrane organelles which are driven by virus-encoded membrane-binding proteins.
- Both structural and nonstructural proteins of the virus contribute to membrane reorganization and viral protein interaction with membranes occurs at several stages of the virus replication cycle.
- The precise role of each protein and of individual domains within each protein in contacting the membrane and initiating its deformation remains work in progress and may vary across the family.
- Certainty has improved considerably for the structural proteins as a number of protein structures now exist, including structures for large molecules and multimeric assemblies such as the Spike protein trimer, obtained by cryoelectron microscopy. Models for the mechanism of protein function based on such structures allows them to be tested.
- Some certainty also applies to the nonstructural proteins in that certain combinations of proteins, notably nonstructural protein (nsp) 3, 4 and 6, can produce membrane deformation and structures that resemble those formed during virus infection. However, the precise contribution of each protein and the role of host proteins in the overall process remains to be determined. Ironically, it is the hydrophobic nature of the proteins concerned that makes them difficult targets for structural biology.
- Regardless of the precise mechanisms of membrane curvature the central role of membrane perturbation in the CoV replication cycle suggests itself as a target for designed intervention. A lack of membrane structures would clearly prevent virus replication but more reasonably even a partial inhibition might result in revelation of the replicative intermediates to the immune system and accelerate virus clearance.
- Study of the membrane reorganization associated with CoV infection is likely to contribute to a greater understanding of membrane biogenesis in general and to offer opportunities for rational design.
- As a universal feature of CoV replication, inhibition of membrane reorganization would likely apply to future zoonotic outbreak strains, as well as, to established and characterized viruses.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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