Nidoviruses
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Chapter 12

Coronavirus Structural Proteins and Virus Assembly

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Coronaviruses are ubiquitous pathogens in vertebrates and cause a variety of diseases, including respiratory infections, gastroenteritis, encephalitis, and hepatitis (194). They are enveloped viruses that contain a positive-strand RNA genome of approximately 30 kb. Their name comes from the typical crown or corona surrounding virions observed by electron microscopy (Fig. 1A). All coronaviruses encode at least three envelope proteins: membrane (M), spike (S), and envelope (E), which use the host cell secretory system for biosynthesis. Some coronaviruses have additional envelope proteins, including the hemagglutinin-esterase (HE) protein found in some group 2 coronaviruses. The other structural protein is the nucleocapsid (N) protein, which encapsidates the RNA genome. Unlike many well-studied enveloped viruses that assemble at the plasma membrane, coronaviruses assemble by budding into the lumen of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (82), a dynamic compartment between the endoplasmic reticulum (ER) and Golgi complex (2). The Golgi complex plays an essential role in processing and sorting of secretory and membrane cargo in all eukaryotic cells, with cargo from the ER entering on the *cis* face and exiting on the *trans* face. The ERGIC (also called the *cis* Golgi network) is at the *cis* face of the Golgi complex and is an active site of protein and lipid sorting. The viral envelope proteins are integral M proteins that are targeted to the ERGIC by independent targeting signals and by interactions with each other. M protein also interacts with the viral nucleocapsid, resulting in virion formation by budding into the lumen of the ERGIC (Fig. 1B). After budding, virions are released from infected cells by exocytosis. The advantage of intracellular assembly is unknown. Here, we review the processing, targeting, and assembly of the coronavirus structural proteins, and the release of assembled virions from infected cells. Several other excellent reviews have also recently covered this topic (39, 118).

STRUCTURAL PROTEINS OF CORONAVIRUS VIRIONS

N Protein

Coronavirus N protein is a multifunctional phosphoprotein that encapsidates the genomic RNA into a helical nucleocapsid within the mature virion (35, 112). The helical nature of the nucleocapsid, which is a common characteristic of negative-stranded animal RNA viruses, is unique for a positive-stranded RNA virus. Through its interactions with the viral RNA, the M protein, and itself, N protein plays important roles in virus assembly (50, 74, 125, 127, 190). The protein is also involved in viral RNA transcription and/or replication (6, 21, 28, 44, 186). Recent studies with coronavirus infectious clones provided direct evidence for N protein in these roles (1, 15, 157, 206). Transmissible gastroenteritis virus (TGEV) and severe acute respiratory syndrome coronavirus (SARS-CoV) N proteins both exhibit RNA chaperone activity in vitro (215). It was suggested that this activity may play a role in template switching during transcription of viral subgenomic transcription, but it remains to be demonstrated that the activity exists in virus-infected cells. The protein may also play a role in viral mRNA translation (171). Additionally, it was recently shown that the N proteins of both mouse hepatitis virus (MHV)-A59 and SARS-CoV are type I interferon antagonists (84, 202).

A three-domain model for the N protein was proposed a number of years ago based on sequence comparison of many MHV strains (Fig. 2) (137).

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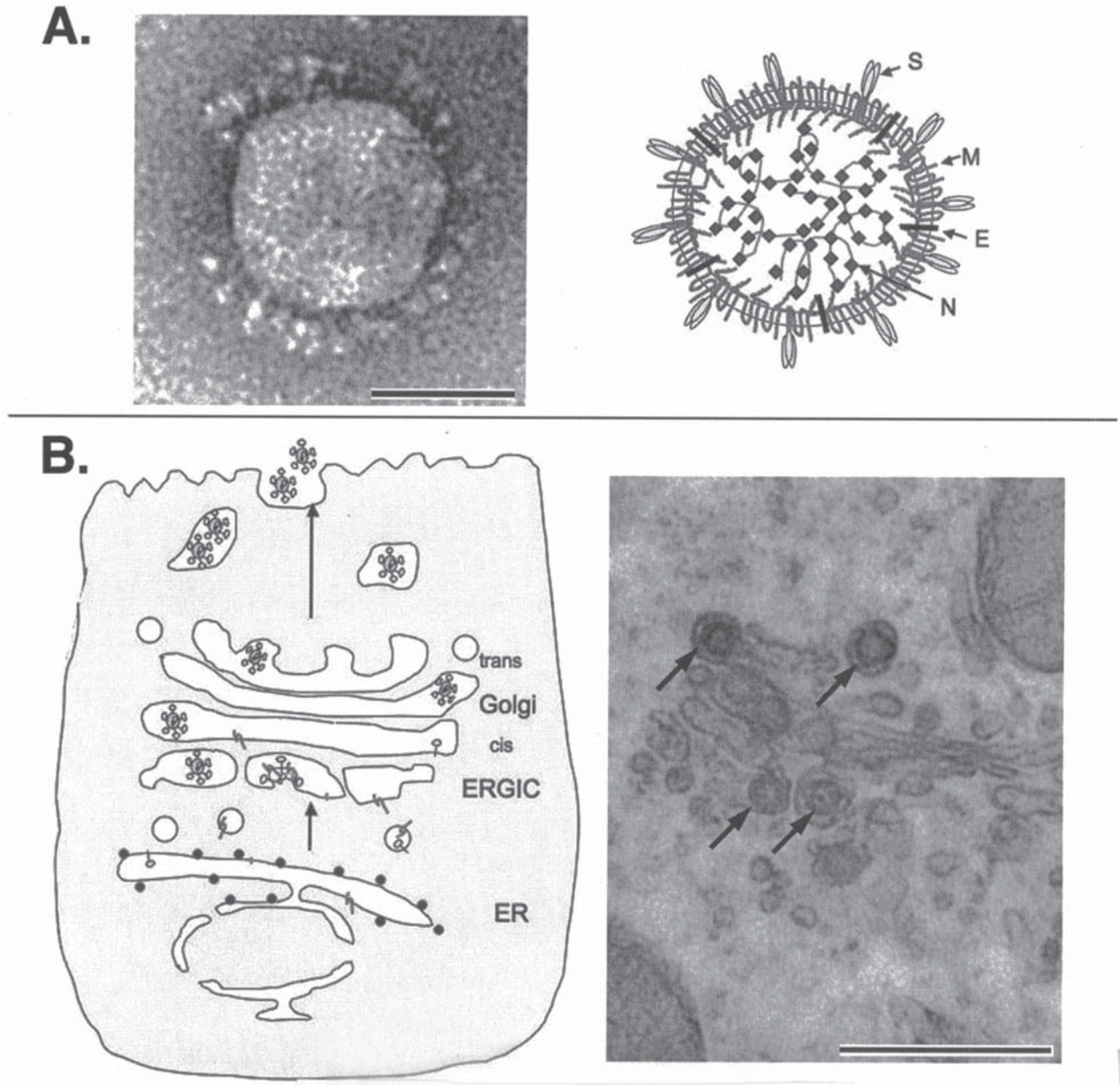

Figure 1. Coronavirus structure and intracellular assembly site. (A) Electron micrograph of purified IBV particle after negative staining (left). Bar, 50 nm. Virion schematic showing the major structural proteins (right). Note that some coronaviruses contain additional E proteins (e.g., HE in some group 2 viruses and several accessory proteins in SARS-CoV). (B) Schematic of virus assembly in cells (left). The E proteins are synthesized in the ER and transported to the ERGIC/Golgi complex. Independent targeting signals and interactions with the other E proteins allow accumulation in the ERGIC, and after interaction with the nucleocapsid, virions bud into the lumen of the ERGIC. They are released from infected cells by exocytosis. The right panel is an electron micrograph of Vero cells infected with IBV, showing a Golgi region with budded virions inside (arrows). Bar, 500 nm.

A serine- and arginine-rich (SR) region in domain II is conserved in different coronavirus N proteins, which is likely involved in some function of the protein. Recently, a modular organization for SARS-CoV N protein was proposed based on results from a number of analytical approaches, including nuclear magnetic resonance (NMR) spectroscopy (19). The new model suggests that the protein consists of two noninteracting domains, the RNA-binding and dimerization domains, with the remainder of the protein being disordered. Bioinformatic analyses suggest that other coronavirus N proteins may have a similar modular organization.

Sequence-specific and nonspecific binding of N protein to RNA has been reported (27, 117, 121, 129, 144, 166, 214). RNA-binding domains have

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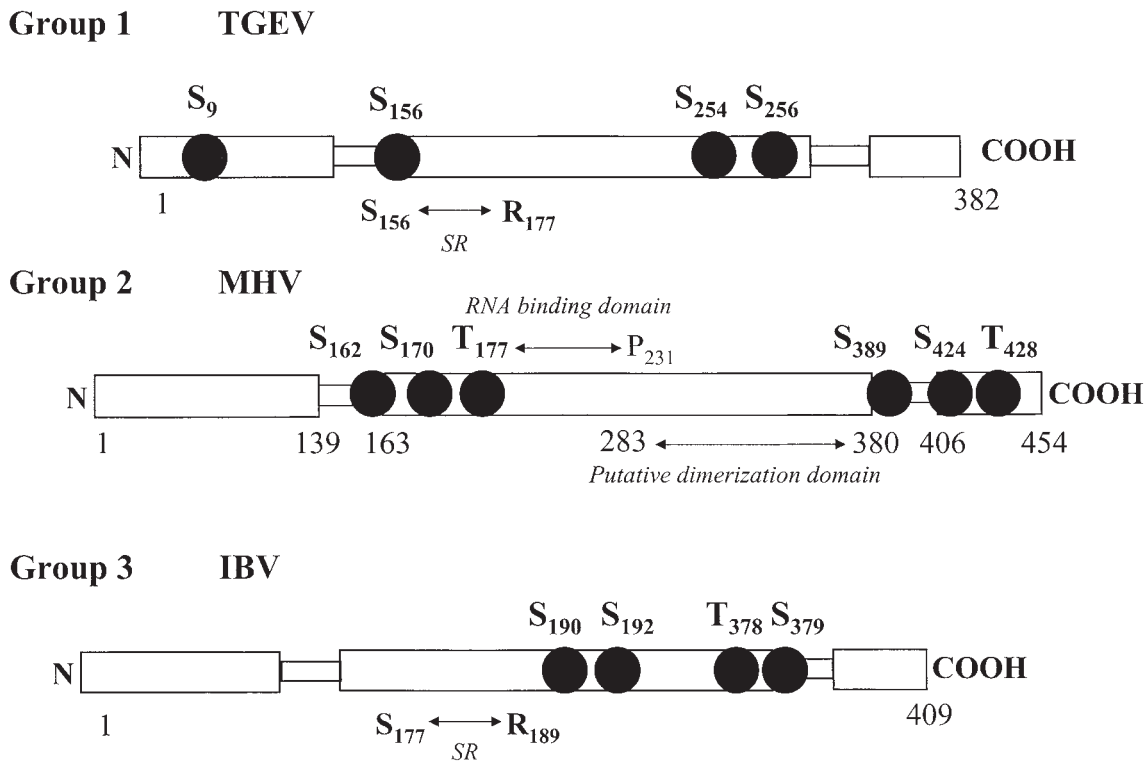

Figure 2. Coronavirus N-protein phosphorylation. A schematic illustrating the three-domain model for coronavirus N proteins with A and B spacer domains (137) is shown at the top. The relative positions of the phosphorylated sites identified on intracellular N protein from TGEV-infected cells (14) and MHV-infected cells (196) and IBV N protein expressed alone (22) are shown below. The positions of the RNA-binding domain that includes the SR region (129) and putative dimerization domain (208) are indicated for MHV. The positions of the SR regions are indicated for TGEV and IBV.

been mapped for MHV, SARS-CoV, and infectious bronchitis virus (IBV) N proteins (51, 72, 129, 214). The domain maps to domain II in MHV N protein, which includes the SR region, whereas the domain is N terminal of the SR region in SARS-CoV and IBV N proteins. The SARS-CoV SR region was reported, based on mammalian two-hybrid screens, to be important for N protein oligomerization and also interaction with M protein, which could be important for assembly of nucleocapsids (62, 63).

All coronavirus N proteins are highly basic, with isoelectric points of 10.3 to 10.7 (90). In addition, all coronavirus N proteins are phosphorylated. The role of phosphorylation is not known, and only very recently were phosphorylated sites identified for IBV, TGEV, and MHV N proteins. There are a large number of potential phosphorylation sites in all coronavirus N proteins, but few of these are actually modified (Fig. 2). Phosphoserines at positions 9, 156, 254, and 256 were identified for the TGEV N protein in virus-infected cells, whereas sites S156 and S256 were identified on N protein in purified virions (14). The sites that are phosphorylated on the IBV N protein expressed with baculovirus in insect Sf9 cells were found to be identical to those present on the N protein expressed alone in Vero cells (22). Data from this study suggest that S190, S192, T378, and S379 are phosphorylated on intracellular IBV N protein. Six residues that cluster on the amino and carboxy ends of the RNA-binding and putative dimerization domains, respectively, are phosphorylated on both intracellular and extracellular virion MHV N proteins (196).

Phosphorylation can regulate protein function, so deciphering the role(s) of phosphorylation of coronavirus N proteins is important. N protein is multifunctional, and phosphorylation could be involved in modulating any of its functions during the virus life cycle, including virus assembly. The RNA-binding affinities of phosphorylated and nonphosphorylated IBV N protein were recently shown to be equivalent when measured by surface plasmon resonance (22). Interestingly, however, the phosphorylated form of the protein bound viral RNA with a higher binding affinity than nonviral RNA. It is possible that the modification alters the structure of the protein and, in turn, presentation of the RNA binding domain(s) that is important for recognition of the packaging signal,

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transcription regulatory sequences, or other signature sequences in the viral RNA(s).

Another modification reported for SARS-CoV N protein is sumoylation at lysine 62 (96). Ubc9, a ubiquitin-conjugating enzyme that participates in sumoylation, may be involved in modifying SARS-CoV N protein, as it was shown to interact with N protein in a yeast two-hybrid screen (52). This modification has not been reported for N proteins from other coronaviruses.

Very recently, structural information has become available for IBV and SARS-CoV N proteins (51, 72, 77, 208). The three-dimensional structures are based on NMR analysis of amino acids 45 to 181 from SARS-CoV N protein and X-ray crystal structures of residues 19 to 162, 219 to 349, and 29 to 160 from IBV-CoV N protein and 270 to 370 from SARS-CoV N protein. The structures of the N-terminal domains of IBV and SARS-CoV N proteins are similar, adopting the same general polypeptide fold consisting of a four- or five-stranded antiparallel β-sheet with a positively charged β-hairpin or loop extension and a hydrophobic core or platform structure (51, 72, 77). High-resolution structures for the C termini of SARS-CoV and IBV N proteins indicate that subunits interact extensively through hydrogen-bonding and hydrophobic interactions to form dimers, strongly suggesting that the oligomer is the major functional unit for the protein (77, 208). NMR structures of the N- and C-terminal domains agree with the crystal structures (18, 19).

M Protein

The M protein is the most abundant protein in the viral envelope, with a short amino terminus exposed on the outside of the virion, three hydrophobic transmembrane domains, and a long carboxy-terminal tail located inside the virion that consists of an amphiphilic region followed by a hydrophilic domain (153) (Fig. 3). The amphiphilic region of the carboxy tail appears to be tightly associated with the membrane (153), which may result in formation of a matrix-like structure that lines the inner virion envelope. All known coronavirus M proteins assume this topology with the exception of that of the group 1 coronavirus TGEV. The TGEV M protein exhibits two topologies in the viral envelope: one population with the amino_{exo}-carboxy_{endo} orientation and another population with an amino_{exo}-carboxy_{exo} orientation (50, 143) (Fig. 3). Most M proteins do not have N-terminal cleaved signal sequences. The group 1 viruses TGEV, feline infectious peritonitis virus, and canine coronavirus do have potentially cleavable signal sequences located at their N termini, but these sequences do not appear to be required for membrane insertion (79, 91, 189). However, the topology of the M proteins lacking the putative signal sequences was not determined, so the signal may be required for proper translocation of the N terminus and the correct orientation of the transmembrane domains.

Coronavirus M proteins are composed of 220 to 262 amino acids, and all are glycosylated on the N-terminal domain. M proteins from group 1 and 3 coronaviruses and SARS-CoV are glycosylated with N-linked oligosaccharides, whereas the M proteins of group 2 viruses exhibit O-linked glycosylation (17, 68, 76, 124, 132, 133, 151, 165). The M protein of MHV strain 2 is modified with both N- and O-linked oligosaccharides (200). The role of glycosylation is unclear, since elimination of the sites for carbohydrate addition in MHV M protein, or swapping the sites from O to N linked, has no effect on targeting or virus production (38). However, the glycosylation

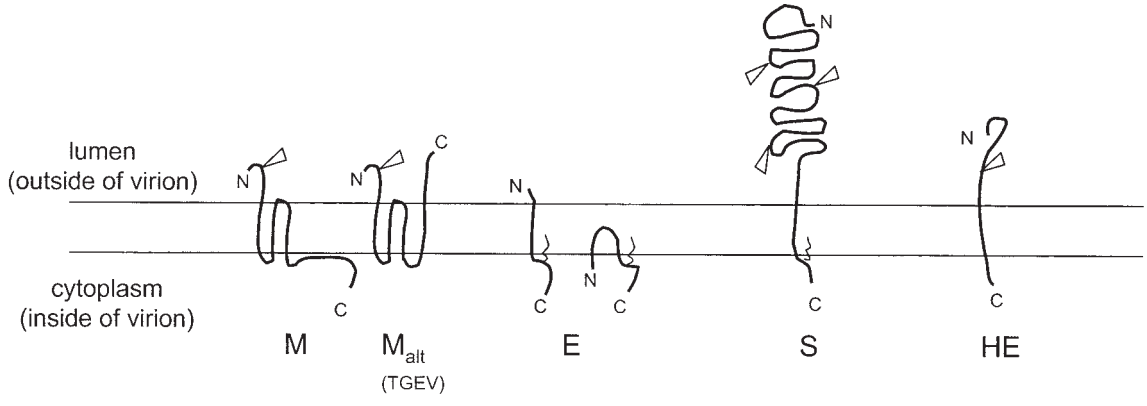

Figure 3. Topology of coronavirus E proteins. Two topologies are shown for M and E proteins, as supported by evidence discussed in the text. Small triangles represent glycosylation but are not meant to indicate the number or type of oligosaccharides, which differ in the proteins from different coronaviruses. S proteins and some E proteins are palmitoylated on their cytoplasmic tails (indicated by the squiggly line).

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status of MHV M protein may contribute to induction of interferon by the virus. Cells infected with MHV containing N-glycosylated M protein induce interferon better than those infected with MHV containing the normal O-glycosylated M protein, and cells infected with MHV containing nonglycosylated M protein are very poor interferon inducers (37). Interestingly, growth of these MHV variants in the liver corresponded to their ability to induce interferon (37). However, an earlier study showed that swapping the N-terminal domain of TGEV (N glycosylated) with that of bovine coronavirus (BCoV) (O glycosylated) had no effect on interferon induction by virus-like particles (VLPs) produced by coexpression of TGEV M and E proteins (7).

E Protein

Coronavirus E proteins are small (76- to 109-amino-acid) integral membrane proteins that lack a cleaved N-terminal signal sequence and have fairly long membrane anchor domains. The protein is a minor structural component of virions but plays an important, not yet fully defined, role in virus production (33, 55, 88, 101, 136, 209). Two topologies for E protein have been proposed, with either one or two transmembrane domains (Fig. 3). Studies on different E proteins agree that the C terminus is cytoplasmic. If the protein spans the membrane once, this would indicate a type III membrane protein with the N terminus translocated across the membrane in the absence of a cleaved signal sequence. IBV E protein appears to adopt this orientation, since the N terminus was accessible to antibody only after permeabilization of Golgi membranes (29). In addition, IBV E protein's hydrophobic region can replace the membrane-spanning domain of a type I membrane protein, showing that it can adopt a transmembrane configuration with an N_{exo}-to-C_{endo} orientation (31). However, several studies have shown that the epitope-tagged N termini of MHV and SARS-CoV E proteins are in the cytoplasm (115, 210). This would result in a protein with two transmembrane domains, or a "hairpin" conformation, with a loop inserted into the cytoplasmic face of the bilayer. Caution must be used in interpreting these results, since extending the N terminus with an epitope tag could block its translocation, altering the topology of the protein. However, synthetic peptides corresponding to the SARS-CoV E protein's hydrophobic region inserted into model membranes adopt the hairpin conformation (3, 81). An epitope tag inserted at the N terminus of IBV E protein allows translocation of the N terminus, whereas the same N-terminal tag on SARS-CoV E protein does not (210), which supports the idea that IBV and SARS-CoV E proteins may adopt different topologies. Perhaps coronavirus E protein can adopt two membrane conformations, each with a specific function. Antibodies that recognize the N termini of MHV and SARS-CoV E proteins will be required to confirm the topology of the native proteins.

Coronavirus E proteins are not glycosylated. The only modification that has been documented is palmitoylation for some coronavirus E proteins. IBV and SARS-CoV E proteins are palmitoylated on cytoplasmic cysteine residues near the hydrophobic segment (31, 98). Palmitoylation of MHV E protein in infected L2 cells was inferred from a mobility shift that is sensitive to alkaline hydroxylamine cleavage (209), but it has not been directly demonstrated. However, another group did not observe the hydroxylamine sensitivity of MHV E protein when expressed by transfection in OST-7 cells (141). The function of palmitoylation is unknown.

Coronavirus E proteins share characteristics with small hydrophobic membrane viroporin proteins of other viruses (60). Viroporins are defined as proteins that alter cellular permeability, and some viroporins are ion channels. Viroporins contain at least one highly hydrophobic domain that forms an amphipathic (-helix in the membrane. The proteins are generally oligomeric, with hydrophobic residues facing the phospholipid bilayer and hydrophilic residues lining the pore (60). Recently, the SARS-CoV and MHV E proteins were shown to exhibit viroporin activity (97, 98, 113). In addition, peptides corresponding to the E proteins from SARS-CoV, MHV, IBV, and human coronavirus strain 229E form cation-specific ion channels in planar bilayer systems (197, 198). Ion channel activity of E proteins would require formation of homo-oligomers. Multimers that are resistant to sodium dodecyl sulfate during electrophoresis have been observed for some coronavirus E proteins, although it is not clear if oligomers form in cells. However, molecular dynamic simulations predict that homo-oligomers are possible for these proteins (182). These predictions are based on E proteins with an N_{exo} and C_{endo} orientation and a single transmembrane domain.

S Protein

S protein extends from the envelope to give coronaviruses their characteristic "crowned" appearance. S protein binds the host cell receptor and mediates virus-to-cell and cell-to-cell fusion (57). S proteins are large, between 1,160 and 1,450 amino acids in length, and are heavily glycosylated to yield 150- to 200-kDa monomers. They are type I membrane proteins, with a cleaved signal sequence, a single

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transmembrane domain, and a short C-terminal tail inside the virion (Fig. 3).

MHV-A59 S protein contains 22 sites for N-linked glycosylation, many of which are used. In addition, there are many intrachain disulfide bonds that are essential for proper folding (134). Homo-oligomerization of coronavirus S proteins has also been reported. Such oligomerization is expected to occur in the ER after folding but prior to export to the Golgi complex, like many other viral envelope proteins that have been examined (45). IBV S protein was reported to form a homodimer or homotrimer (16). The shed S1 subunit of MHV S protein behaves as a homodimer (94), although the S2 subunit appeared to run as a trimer on immunoblots (58). Homotrimers of TGEV S protein were shown to form early after synthesis, prior to Golgi-specific carbohydrate processing (43). Trimers of SARS S proteins have also been reported (95, 124). MHV S protein is slow to fold, and "older" S molecules interact with "newer" M molecules, suggesting that S protein folding rates influence its ER export and assembly (135). However, this does not seem to be the case for BCoV, where newly synthesized S protein interacted immediately with newly synthesized M protein (and HE; see below) (131). In addition to glycosylation, S proteins are palmitoylated on one or more cysteine residues in the cytoplasmic domain near the transmembrane domain (9, 168, 185). These cysteine residues have been implicated in cell-to-cell fusion by MHV S (9, 20). A recent study showed that palmitoylation of MHV S protein is important for interaction of S protein with M protein, and assembly of infectious virus (176).

Some coronavirus S proteins are cleaved into S1 and S2 subunits by furin or a related enzyme in the Golgi complex (39). S proteins of group 1 coronaviruses and SARS-CoV lack a furin cleavage site. Although SARS-CoV S protein is not cleaved during biogenesis, cleavage upon entry by endosomal proteases is required for efficient infection (71, 162). Cleavage of MHV S protein is not essential for infectivity but may enhance cell-to-cell fusion (41).

HE Protein

Some group 2 coronaviruses express another structural protein, HE, that is anchored in the envelope as a second spike. HE protein is synthesized as a 42.5-kDa protein that is subsequently glycosylated to 65-kDa disulfide-linked dimers (67). It is a type I membrane protein with a cleaved signal sequence, a single transmembrane domain, and a short cytoplasmic tail (Fig. 3). HE dimers associate with M and S proteins in virus-infected cells (131). Proper oligomerization appears to be necessary for incorporation of HE into these complexes.

HE hydrolyzes O-acetylated sialic acid on oligosaccharides to which S protein binds, presumably allowing reversible attachment. Interestingly, the specificity of HE differs in different coronaviruses, likely due to host species variability in sialic acid modifications (164). In MHV, strains expressing functional HE are more neuropathic than those that do not express HE (80). However, the functional gene is rapidly lost with passage in cell culture (100).

TARGETING OF VIRAL STRUCTURAL PROTEINS

Targeting of N Protein

N protein is synthesized in the cytoplasm, where it interacts with newly synthesized viral genomes to form nucleocapsids. MHV N protein can also be detected at RNA replication sites at membranes early in infection (12, 186). IBV N protein was reported to localize to nucleoli as well as the cytoplasm, both in infected and in transfected cells (65). Hiscox and colleagues have shown that IBV N protein interacts with cellular nucleolar proteins, and IBV infection induces morphological changes in nucleoli (23, 46). The same group showed that N proteins of TGEV and MHV could also be found in nucleoli of transfected cells (199). The localization of N protein to nucleoli could influence host ribosome biogenesis and cell growth and division. However, most investigators have not detected nuclear or nucleolar localization of the N protein in cells infected with TGEV or MHV (14, 163). In addition, several groups have reported that SARS-CoV N protein is cytoplasmic, even when nuclear export is blocked with leptomycin B (154, 169, 204). Thus, the localization of coronavirus N proteins to nuclear subdomains does not appear to be a universal property. A recent report showing that individually expressed fragments of SARS-CoV N protein did localize to the nucleus or nucleolus (177) raises the possibility that at least some of the discrepancies reported for targeting of coronavirus N proteins could be due to proteolytic processing in some situations.

Targeting of M Protein

MHV, IBV, TGEV, feline infectious peritonitis virus, BCoV, and SARS-CoV M proteins are all targeted to the Golgi region when expressed individually from cDNA (82, 110, 124, 131, 152). Given the abundance of M protein in viral envelopes and its targeting to the Golgi region, it was surprising when localization at the electron microscopic level showed

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that M protein does not, on its own, determine the site of virus assembly. In transfected cells, IBV M protein is localized to the *cis* side of the Golgi stack (109), whereas MHV M protein reaches the *trans* side of the organelle (82). Thus, neither IBV nor MHV M protein is retained in the ERGIC, the site of assembly. Association with other viral proteins is believed to restrict trafficking of the M protein, to collect it in the ERGIC for assembly.

Golgi region targeting of coronavirus M proteins has been most extensively studied for MHV and IBV. For IBV M protein, a signal for Golgi localization was identified in the first of the three transmembrane domains (110). Deletion of the second and third transmembrane domains has no effect on Golgi localization, but deletion of the first and second membrane spans results in transport to the plasma membrane. Deletion of most of the cytoplasmic tail also has no effect on Golgi localization. The first transmembrane domain of IBV M protein retains a plasma membrane reporter protein, vesicular stomatitis virus G protein (VSV-G), in the *cis* Golgi region when it is inserted in place of the normal VSV-G transmembrane domain (170). The sequence appears to function as a true retention signal, since VSV-G containing the IBV M protein first transmembrane domain (Gm1) forms large, detergent-insoluble oligomers upon arrival at the Golgi complex (195). The critical sequence in the transmembrane domain was mapped to polar uncharged residues that line one face of a predicted ∝-helix. Mutation of any one of these four polar residues results in transport of the Gm1 chimera to the cell surface (108). Although quite surprising at the time, Golgi resident proteins were subsequently shown to use targeting information within their transmembrane domains as well (25). However, there is no sequence conservation or motifs that are shared within these proteins. A popular model for the mechanism of Golgi protein localization originated from the observation that some Golgi membrane proteins had shorter predicted transmembrane domains than did plasma membrane proteins (123). Membrane thickness, commonly believed to increase from the ER to the plasma membrane due to a cholesterol gradient, could thus mediate localization of Golgi membrane proteins due to a membrane partitioning effect (13). However, it was subsequently shown that transmembrane domains themselves determine the membrane thickness, rather than the other way around (120). Other factors must therefore contribute to efficient localization of Golgi membrane proteins, although the lipid composition of Golgi membranes is likely to play a role.

MHV M protein requires sequences in both its cytoplasmic tail and its transmembrane domains for Golgi localization. When the first and second transmembrane domains of MHV M protein are deleted, the protein is transported past the Golgi complex to endosomes and lysosomes, suggesting that this region of the protein contributes to Golgi localization (5, 102). The cytoplasmic tail of MHV M is essential but not sufficient for Golgi targeting, with C-terminal residues playing a key role (5, 102). In addition, other regions of MHV M protein contribute to targeting as well (4). The first transmembrane domain of MHV M protein is unable to retain VSV-G in the Golgi complex, confirming that IBV and MHV M proteins use different mechanisms for steady-state localization (108). Perhaps the differences in the Golgi targeting domains for IBV and MHV M proteins are not so surprising, considering that the two M proteins are targeted to opposite faces of the Golgi complex.

Targeting of E Protein

Different investigators reported that IBV E protein is targeted to the Golgi region (29) or to the ER (99) when expressed from cDNA. The latter study implicated a dibasic ER retrieval signal in targeting of IBV E protein containing a C-terminal epitope tag (99). By immunoelectron microscopy, untagged IBV E protein is localized to *cis* and *medial* Golgi membranes when expressed in BHK-21 cells (30). It is also localized to the Golgi region in many other cell types at the light microscopic level. One possible explanation for the different results for IBV E protein targeting is the use of epitope tags or different expression systems. The Golgi targeting reported by Corse and Machamer depends on a central region of the cytoplasmic tail (31). This region shares some homology with the cytoplasmic tail of the G1 protein from Uukuniemi virus (a bunyavirus), which is also targeted to Golgi membranes (145). The targeting signal in the cytoplasmic tail of IBV E protein is likely to interact with cellular Golgi proteins, but these have not yet been identified.

MHV E protein is localized to the ERGIC when expressed independently (141). The same study showed that overexpression of MHV E induces smooth, convoluted membrane accumulations, presumably derived from the ER. Targeting signals have not been reported.

The localization of SARS-CoV E protein when expressed from cDNA may vary with the cell type and expression system. Using a vaccinia virus T7 expression system, epitope-tagged SARS-CoV E protein is localized in the ER in HeLa cells, but in BHK-21 cells, both tagged and untagged SARS-CoV E proteins are localized to the Golgi region (98). Using a Semliki Forest virus expression system, tagged

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SARS-CoV E protein was reported to partially overlap with ER markers in BHK-21 cells (124). However, using a plasmid expression system, tagged SARS-CoV E protein is localized to the Golgi region in BHK-21 cells (105). Mutations in the hydrophobic region of SARS-CoV E protein disrupt membrane localization (98), but specific targeting signals for ER or Golgi localization have not yet been reported. These collected findings warrant caution in determining intracellular localization of coronavirus E proteins when expressed from cDNA, as well as ER and Golgi targeting signals.

Targeting of S Protein

Some coronavirus S proteins contain information in their cytoplasmic tails that contributes to targeting. IBV and other group 3 coronaviruses possess canonical dilysine ER retrieval signals (104). This type of signal requires a lysine residue in the −3 and the −4 or −5 position from the C terminus. The dilysine signal binds a coat complex known as COPI, which coats vesicles that form on Golgi membranes that are subsequently targeted to the ER (175). The signal is found on ER and ERGIC resident membrane proteins and is required for retrieval if they escape these compartments. The dilysine signal on IBV S protein ($KKSV_{COOH}$) contributes to its localization near the virus assembly site, although at high expression levels the machinery is saturated and IBV S protein reaches the plasma membrane. When a mutation in the dilysine signal was introduced into an infectious clone of IBV, the resulting virus had a growth defect, including premature formation of syncytia (205). Interestingly, the S proteins of group 1 coronaviruses and SARS-CoV contain a related dibasic signal, $KXHXX_{COOH}$. In SARS-CoV S protein, this dibasic signal functions similarly to the dilysine signal in IBV S protein, although less efficiently (119). Mutation of the dibasic signal results in faster trafficking of SARS-CoV S protein through the secretory pathway, which, in turn, prevents efficient interaction with SARS-CoV M protein when these proteins are coexpressed (119). Thus, cycling between the Golgi region and ER induced by the dibasic signal may be critical for allowing sufficient opportunity for SARS-CoV S and M proteins to interact. Group 2 coronavirus S proteins, including MHV S protein, lack a dibasic signal in their cytoplasmic tails. Slower folding and assembly of S protein, or more robust interaction with M protein, might preclude the necessity for ER retrieval in S proteins from group 2 coronaviruses. It is currently unknown whether the presence or absence of an ER retrieval signal on S proteins influences virus pathogenesis.

TGEV S protein contains the $KXHXX_{COOH}$ dibasic signal found on the SARS-CoV S protein mentioned above. However, Schwegmann-Wessels et al. reported that TGEV S protein was localized to the ER by a tyrosine-based motif found upstream of the dibasic signal (158). In a different study, the last 11 amino acids of the TGEV S tail (containing both the dibasic signal and the tyrosine motif) were able to retain a reporter construct in the ERGIC, and this localization was dependent only on the dibasic motif (104). The different results from the two groups could reflect different cell types, expression systems, or the fact that the chimeric reporter construct does not accurately reflect the behavior of the full-length protein. Alternatively, the tyrosine-based motif could contribute to rapid endocytosis of TGEV S protein from the plasma membrane, which would have been missed in the steady-state experiments that were used to analyze the localization of full-length TGEV S protein (158).

IBV S protein contains a second targeting signal in its cytoplasmic tail: a canonical endocytosis signal (YTTF) upstream of its dilysine signal. This type of sequence binds the AP2 adaptor complex that, in turn, binds clathrin to induce endocytosis (8). IBV S protein is endocytosed from the plasma membrane in both infected and transfected cells (205). Mutation of the tyrosine residue blocks endocytosis, and mutation of both ER retrieval and endocytosis signals allows accumulation of IBV S protein at the surface of transfected cells, with a concomitant increase in syncytium formation. Interestingly, recombinant virus could not be recovered when the tyrosine in the endocytosis signal was mutated (205), suggesting that too much IBV S protein at the surface of infected cells is incompatible with virus replication, or that the tyrosine residue plays another important role in the virus life cycle.

SARS-CoV Accessory Proteins Present in Virions

Several proteins encoded by the "group-specific" open reading frames in SARS-CoV are incorporated into virions. In other coronaviruses, these accessory proteins are expressed in infected cells, but none have been reported to be structural proteins. SARS-CoV 3a protein has a topology similar to M protein, with three membrane-spanning domains and an O-glycosylated N terminus (133). It is the largest of the SARS-CoV accessory proteins, with 274 amino acids. Several reports have shown that SARS-CoV 3a protein is present in purified virions (75, 161). One group reported that when it is expressed from cDNA, SARS-CoV 3a protein is transported to the plasma membrane and is endocytosed (173). However, deletion of several motifs

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that are often involved in endocytosis actually blocked surface delivery. Other investigators have reported that SARS-CoV 3a protein is localized to the Golgi region (211) or plasma membrane (106) in infected cells, and to the Golgi region (133) or the ER (93) in transfected cells.

SARS-CoV 7a protein is a type I membrane protein of 122 amino acids, with a cleaved signal sequence and a single transmembrane domain (54). Like the 3a protein, SARS-CoV 7a protein is incorporated into virions (70). The structure of the 7a ectodomain shares features with the immunoglobulin superfamily (128). The protein contains a canonical ER retrieval signal at its C terminus ($KRKTE_{COOH}$) and when expressed from cDNA is localized in the ER and ERGIC region. However, both the transmembrane and cytoplasmic domains are required to retain a reporter protein in the ER-Golgi region (128).

SARS-CoV 7b protein is a small hydrophobic integral membrane protein of 44 amino acids that is produced from subgenomic RNA7 by leaky ribosome scanning (156). The C terminus of SARS-CoV 7b protein is in the cytoplasm, but it is not clear whether the protein spans the bilayer or is inserted as a hairpin. The protein is targeted to the Golgi region in both transfected and infected cells and is incorporated into virions (156).

It will be important to determine the level of these accessory proteins incorporated into SARS-CoV virions. Are only a few molecules present, or are they present at substantial levels? Deletion of all of the accessory proteins from the SARS-CoV genome has no effect on virus production from cultured cells (207), so none of the three proteins discussed above are essential for virus production. Like accessory proteins from other coronaviruses, SARS-CoV accessory proteins are believed to impact the host antiviral response. SARS-CoV 3a protein induces apoptosis when expressed in Vero E6 cells (93) and up-regulates fibrinogen expression in lung epithelium (174). SARS-CoV 7a protein has been reported to induce apoptosis (172), to block cell cycle progression (212), and to inhibit host protein synthesis and activate p38 mitogen-activated protein kinase (85). It will be important to determine if these any of these reported activities depend on incorporation of the accessory proteins into virions.

VIRUS ASSEMBLY

Coronaviruses assemble at internal membranes of the ERGIC (82, 179). Exactly what determines the site of assembly is still not fully understood, although the localization of E proteins near this compartment certainly contributes to the process. S, M, E, and N proteins all form complexes that through multiple interactions presumably drive assembly. Interestingly, only M and E proteins are required for assembly of the viral envelope. Coexpression of M and E proteins in the absence of the other viral components is sufficient for assembly of MHV, IBV, BCoV, and SARS-CoV VLPs (7, 10, 29, 69, 122, 188). S protein is incorporated into VLPs when coexpressed with M and E proteins (10, 188). One exception is a report indicating that SARS-CoV M and N proteins are sufficient and required for VLP assembly (73), although this study did not analyze the release of VLPs. Thus, in contrast to most enveloped viruses, coronavirus envelope proteins are able to assemble and form particles independent of nucleocapsids. The unusual structure of M protein (membrane spanning with a cytoplasmic tail that is also tightly associated with the bilayer), coupled with its ability to oligomerize, may promote nucleocapsid-independent budding in the presence of E protein, which might increase membrane curvature. The unique lipid composition of the ERGIC (24) may also contribute to this unusual property of nucleocapsid-independent budding.

Role of M Protein in Assembly

M protein is a major player in assembly of the viral envelope through interactions with itself, the other E proteins, and the nucleocapsid (40, 42, 47, 87, 131, 135). M protein is the most abundant protein in the mature virion, and VLPs consist primarily of M protein with only a few molecules of E protein. Thus, M-M protein interactions are thought to provide the overall scaffold for assembly of the envelope. M protein forms large multimeric complexes, and M-M lateral interactions in the membrane are thought to be primarily responsible for assembly of the viral envelope, even though the protein is not capable of driving the process when expressed alone (103).

Interactions between M protein molecules appear to be mediated by multiple domains. MHV M proteins with deletions in the luminal domain, transmembrane domains, the amphipathic domain, or the hydrophilic C-terminal tail are unable to assemble into VLPs (38). However, when the mutants were analyzed for the ability to associate with assembly-competent M proteins, only mutant proteins with replacement of the three transmembrane domains were not incorporated into VLPs, suggesting that M-M interactions are mediated through their transmembrane domains (42). Targeted RNA recombination demonstrated that complete virions are more tolerant of changes in the C terminus of the M protein tail than VLPs, since some mutants could be

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assembled into virions. Both VLP and virus assembly are sensitive to deletion and changes of the two C-terminal residues (38, 87, 191).

E-M Protein Interactions

The role of E protein in virus assembly is far from understood, but it is clear that the protein is important for virus assembly. As mentioned above, VLP assembly is dependent on coexpression of E and M proteins, which are sufficient for their production and release (7, 10, 29, 122, 188). This strongly implies that the two proteins must interact at some level. Interaction between IBV E and M proteins was demonstrated in IBV-infected cells and in transfected cells by cross-linking and coimmunoprecipitation (32). The IBV E protein cytoplasmic tail mediates its interaction with the cytoplasmic tail of M protein (32, 99). M protein is far more abundant in virions and VLPs than E protein, but expression of M protein alone does not produce VLPs. As originally proposed by Vennema and colleagues (188), E protein could induce curvature at precise sites in a lattice composed of M protein or could promote particle scission. However, E protein is not a universal requirement for coronavirus particle formation (see below). Another observation is that E protein-containing vesicles are released from cells when E protein is expressed alone (29, 114). The significance of this is not known, but it suggests that the activity provided by the protein can function in the absence of M protein or the other viral proteins.

Deletion of the E protein gene from MHV results in severely crippled virus (88), whereas removal of the protein from TGEV-CoV blocks virus production (33, 136). SARS-CoV E protein is important for virus production, but not absolutely essential, since a mutant virus lacking the gene yielded titers ranging from 20- to 200-fold less than the wild-type virus (depending on the cell type used to grow the virus) (36). The specificity of E protein interaction with the M protein was recently investigated by replacing MHV E protein with the heterologous counterpart from other coronaviruses (86). MHV E protein could be replaced by E protein from the related group 2 viruses BCoV and SARS-CoV and, surprisingly, by E protein from group 3 IBV. However, E protein from group 1 TGEV could not substitute for MHV E protein. Even though there is little sequence homology between MHV and IBV E proteins, IBV E protein was incorporated into the complemented MHV virions, suggesting a specific interaction with M protein, or at least a precise role in particle formation (86).

The importance of the MHV E protein transmembrane domain in assembly has recently been illustrated. Alanine scanning insertion mutagenesis was used to examine the effect of disruption of the domain and gain insight into its possible function beyond serving to anchor the protein in the membrane (203). MHV mutants with insertions in the transmembrane domain exhibited a small-plaque phenotype and were significantly crippled in their growth. The most striking difference between the crippled viruses and recovered viruses that grew similarly to the wild-type virus was the positions of four hydrophilic polar residues along one face of the predicted α-helix. The positions of these residues on one face of the predicted α-helix are conserved in essentially all E proteins, suggesting that this is a functionally important feature of the proteins. These residues and their positioning along one face of the transmembrane α-helix may be essential for the overall structure of the transmembrane domain or of the entire protein. The transmembrane domain could play a role in protein-protein interactions or some other function that impacts the protein's ion channel activity, which, in turn, plays a role in assembly of the viral envelope and/or release of assembled virions as they mature through the exocytic pathway via transport vesicles. A second study clearly demonstrated that the transmembrane domain of E protein is required for efficient release of IBV (111) (see "Release of Virus from Infected Cells" below).

S-M Protein Interactions

Most coronavirus S proteins are transported past the virus assembly site, and interaction with M protein is required for incorporation into virions. Recently, the requirement for incorporation of S protein into MHV virions was mapped to the juxtamembrane cysteine-rich and central regions of the cytoplasmic tail (11, 201). Palmitoylation of multiple cysteine residues of the MHV S tail is required for efficient interaction with the M protein (176). Dissection of the domain(s) of M protein that interacts with S protein is incomplete and has been studied only by coimmunoprecipitation (40). Deletion of the amphipathic domain has a severe affect on M-S interaction, whereas deletion of the amino and extreme carboxy domains does not.

N-M Protein Interactions

Interactions between M and N proteins have been analyzed both in vitro and by reverse genetics. For TGEV, an in vitro binding assay using a panel of M protein substitution and deletion mutants and purified nucleocapsids mapped a 16-amino-acid domain located 10 residues from the C terminus of

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the protein that is responsible for M-nucleocapsid interaction (49). Two different amino acid stretches in the central region (residues 211 to 254 and 168 to 208) of SARS-CoV N protein were reported to be required for interaction with M protein (53, 63). These results are based primarily on in vitro pull-down assays and mammalian two-hybrid analysis; thus, further studies are required to validate the significance of these interactions in SARS-CoV-infected cells.

More recently, genetic analysis has provided evidence for MHV N-M protein interactions and new insight about the interactions. Initial attempts to isolate by recombination a virus lacking the two C-terminal amino acids in M protein suggested that the mutation was lethal; however, virus lacking these residues was subsequently isolated using more stringent host range selection (38, 87). The recovered MΔ2 viruses had an extremely defective phenotype, with very small plaques and low titers, but viruses were recovered after several passages with second-site changes in the M or N proteins, some of which were shown to compensate for deletion of the terminal two amino acids (87). The second-site changes mapped to regions in the C terminus of the M or N proteins.

Two additional studies showed that negatively charged amino acids in the C terminus of MHV-A59 N protein are important for virus assembly. Two aspartic acid residues (D440 and D441) in N protein were independently identified by two groups as residues necessary for virus assembly (74, 191). One of the studies identified second-site suppressor changes that compensated for mutations in the D440 and D441 residues, which provides strong evidence for genetic cross talk between the two proteins (74), whereas the other study identified compensatory changes only in the C terminus of N protein (191). Replacement of the penultimate positively charged R227 in MHV M protein with negative charges resulted in very crippled virus. Adaptive second-site changes restoring growth to the R227A mutant were identified in M protein, changes that were identical to those M protein alterations restoring growth to the D440A-D441A mutant virus (191). It is interesting that when key charged residues (D440 and D441 in N protein or R227 in M protein) are modified, these independently give rise to overlapping second-site suppressor or adaptive changes in the same region of M protein. Results from these studies strongly argue that M-N interactions are complex, indicating that more than just the single R227 and D440-D441 charges are important. This idea is supported further by the fact that reciprocal exchange of the charges between the two proteins was unsuccessful (74, 191).

M-Nucleocapsid and M-RNA Interactions

A number of early studies demonstrated that M proteins from different coronaviruses copurified with nucleocapsids when purified virions were disrupted with nonionic detergents in low-salt buffers (59, 89, 193). More detailed analysis of MHV showed that M protein interacts with nucleocapsids in a temperature-dependent manner when virions were solubilized with nonionic detergent, which appeared to be mediated by M-RNA binding (167). More recent studies demonstrated that TGEV M protein is part of an internal virion core surrounding the helical ribonucleoprotein complex (47, 142).

Interaction between M protein and nucleocapsids containing genome-length RNA has been demonstrated for MHV (126). N protein was associated with all viral RNAs, whereas antibodies specific for M protein only coimmunoprecipitated N protein complexed with genomic RNA. The interaction between M and the N-genomic RNA complexes is dependent on the presence of the packaging signal (see below) that is located in gene 1b of the genome (127). Significantly, the packaging signal was shown to mediate selective interaction between M protein and viral RNA. Coexpression of M and E proteins with a reporter RNA containing the packaging signal was sufficient for selective packaging of the RNA into VLPs without expression of N protein (125). It is interesting that a number of studies over the years have noted potential M-RNA interactions. The suggestion from the latest studies that a viral envelope protein contributes to the selective packaging of genomic RNA is novel. Future studies that focus on further characterization of the M-RNA interaction and mechanistically how it contributes to selectivity of packaging are warranted.

Packaging Signals

Packaging signals specify selective encapsidation of viral genomic RNA into virions. Coronaviruses synthesize a nested set of 3′ coterminal subgenomic RNAs that share a common 5′ leader sequence, but most package only the full-length genomic RNA in the mature virion. Subgenomic RNAs have been detected in purified BCoV, TGEV, and IBV (66, 116, 159, 160, 213). However, recent analysis demonstrated that subgenomic mRNAs are not present in extensively purified TGEV virions (48). Packaging signals have been identified for a number of coronaviruses. The MHV signal was the first to be identified and requires a 61-nucleotide stem-loop structure that is present approximately 21 kb from the 5′ end of the genome in gene 1b for RNA packaging (56). A similar sequence in BCoV is a functional packaging signal

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(26). The packaging signal for TGEV is located within the first 649 nucleotides at the 5′ end of the genome (48). Sequences within the 5′ and/or 3′ untranslated region of IBV are thought to be required for packaging, but signals necessary for RNA replication are also located in these regions, making it difficult to completely distinguish between these functions (34).

RELEASE OF VIRUS FROM INFECTED CELLS

Post-Golgi Transport of Constitutive Cargo

Coronaviruses are believed to follow the constitutive secretory pathway for exocytosis. Originally, it was thought that coronavirus release might be an unusual type of exocytosis, since virions are larger (~100 nm) than typical secretory vesicles (80 nm). Large vacuoles containing virions are usually greater than 500 nm in diameter. However, recent characterization of post-Golgi carriers used by cellular cargo (described below) suggests that virus release could follow a normal cellular route.

Until recently, constitutive transport of cargo from the Golgi complex to the plasma membrane was believed to follow a conventional vesicle-mediated pathway similar to that of cargo leaving the Golgi complex that is diverted to other destinations, like lysosomes. The cargo that is sorted at the *trans* Golgi network for other destinations is concentrated and requires the coat protein clathrin and associated adaptor proteins (183). However, constitutive cargo leaving the Golgi complex en route to the plasma membrane has been shown to exit in large, pleomorphic carriers lacking a coat (107). Experiments following fluorescent protein-tagged cargo in live cells identified transport carriers leaving the Golgi complex that are tubular with saccular regions and quite long, averaging between 1 and 2 μm in length (64, 139, 178). The molecular requirements for formation and fusion of these post-Golgi carriers are currently under active study. It was reported that small and large cargo (VSV-G and procollagen) can exit the Golgi complex in the same transport carriers (138). However, it is not known if large spherical cargo like coronaviruses can be packaged into the same type of transport carriers as other cargo, and whether these large cargoes require additional cellular machinery.

Coronavirus Exocytosis

Few studies have directly examined coronavirus release. Drugs that perturb the late secretory pathway (e.g., monensin and weak bases) also block release of TGEV (155). A characteristic maturation of TGEV virions that occurs during transport was also reported in this study. Mature particles (which are not present in monensin-treated cells) are smaller and more condensed than immature particles. The only other study to examine exocytosis of coronavirions was one that analyzed MHV in cells that perform regulated secretion (AtT20 cells). It was demonstrated that virions are sorted away from regulated secretory products at the Golgi complex (181), suggesting that MHV release follows a constitutive pathway. A characteristic morphological change was also noted for virions in this study; empty-looking particles are present in the Golgi region, whereas those present in post-Golgi vacuoles are more electron dense.

Another factor in virus release may be the significant cellular alterations that occur in infected cells. Late in infection, the Golgi complex is disrupted, with fragmentation of the Golgi ribbon and dispersion of stacks (92, 180). This may hinder transport of virions through the Golgi complex or their incorporation into secretory vesicles. This idea is supported by the observation that late in infection, MHV budding was shown to occur in ER membranes, where large numbers of virions accumulated (179). In coronaviruses that induce syncytia, cellular alternations may also significantly impact virus release. After cell fusion, the microtubule network is rearranged and Golgi complexes from individual cells move together to form one organelle near the center of the syncytium (92). Given these alterations, post-Golgi transport intermediates containing virions may have a greater distance to travel to reach the plasma membrane, and they might not have the appropriate microtubule tracks for efficient delivery.

Polarized Release of Coronaviruses from Epithelial Cells

Polarized epithelial cells are the first cells infected during coronavirus infection. These cells line all internal and external surfaces of the body and are characterized by two distinct plasma membrane domains (apical and basolateral) separated by tight junctions. The apical or basolateral localization of the virus receptor determines the site of coronavirus entry in polarized epithelial cells. In respiratory epithelial cells, SARS-CoV and human coronavirus strain 229E preferentially enter and are exocytosed from the apical surface (78, 184, 192). In a similar way, TGEV preferentially enters and exits from the apical surface of a polarized porcine kidney line (147). However, although MHV also preferentially infects cells from the apical surface, the polarity of release varies depending on the cell line. In a mouse kidney line and a porcine kidney line (expressing the MHV receptor), release occurs preferentially from

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the basolateral side, whereas in a canine kidney line (also expressing the receptor), release is apical (146, 149, 150).

The polarity of release of infectious virus will impact the ability to generate a localized versus systemic infection. How is sorting of virus-containing exocytic vesicles achieved? Sorting into vesicles destined for the apical or basolateral surface requires signals in the cargo itself and occurs in the last Golgi compartment (the *trans* Golgi network) in simple epithelia (130). Since coronaviruses assemble by budding into the ERGIC, these signals would have to be present in intact virions as they move into the *trans* Golgi network. S protein projects from the surface of virions and could be recognized by cellular sorting machinery. However, one study showed that MHV S protein was not involved in determining the polarized release of virus (148). Thus, the mechanism for polarized sorting of virions into transport carriers destined for different plasma membrane domains is still not understood.

Role for Coronavirus E Protein in Virion Exocytosis?

Recent evidence suggests that coronavirus E proteins form cation-specific ion channels when reconstituted into planar lipid bilayers (197, 198). This activity may be important during infection, since drugs that block the in vitro channel activity reduced infectivity in an E protein-dependent manner (197). The E protein is absolutely required for replication of TGEV (136) but is not essential for MHV or SARS-CoV (36, 88). There may be more than one function for E protein, and these functions may be virus and cell type specific. Although a specific effect on release of virions was not reported, cells infected with a recombinant SARS-CoV lacking E protein have reduced numbers of intracellular mature virions relative to those infected with wild-type virus, and intracellular vacuoles contain potentially aborted assembly intermediates or degraded material (36).

A recent study on IBV supports the idea that the transmembrane domain of E protein is critical for exocytosis of infectious virions. IBV E protein with a complete replacement of its transmembrane domain (called EG3) is targeted to the Golgi region, is palmitoylated, and assembles with M protein to form particles normally (31, 32). However, cells infected with a recombinant virus encoding EG3 instead of E protein are significantly deficient in release of infectious particles (111). Examination of infected cells by electron microscopy showed the accumulation of large, virion-containing vacuoles, many of which contained degraded structures (Fig. 4A). Most S protein in purified particles released from cells infeated with IBV encoding EG-3 is cleaved near the surface of the virion envelope, resulting in noninfectious particles. These data suggest that the transmembrane domain of IBV E protein is required for exocytosis of intact virions. Replacing the transmembrane domain of an ion channel should inactivate it, so channel activity may be required to promote fusion of transport carriers containing virions with the plasma membrane, or to prevent their fusion with lysosomes. Alternatively, the transmembrane domain of E protein could also promote interactions with cellular trafficking machinery. These potential functions for E protein would be required postassembly, and they suggest an additional nonstructural role for the protein (Fig. 4B). E protein is expressed in excess in infected cells relative to what is incorporated into virions, so an additional role(s) as a nonstructural protein is possible.

Why would coronaviruses need a protein that alters intracellular ion concentrations (or interacts with trafficking machinery) to promote release of virions if exocytosis follows normal cellular pathways? Perhaps the sheer number of particles per transport carrier or their spherical structure prevents the carriers from moving normally along microtubules. Another possibility is that the cell recognizes the carriers containing virions as abnormal and targets them for fusion with lysosomes. Rapid progress in this area of coronavirus research is expected.

UNANSWERED QUESTIONS

One of the biggest remaining questions for coronavirus assembly is why these viruses assemble at intracellular membranes. Although most well-studied enveloped viruses bud from the plasma membrane, there are a number of enveloped viruses that assemble by budding into intracellular compartments (61). What are the advantages that compensate for the complication of virion exocytosis after budding? One possibility is that complete assembly inside the cell helps the virus "hide" from the immune system. However, since cytotoxic T cells (the predominant antiviral defense arm of the immune system) recognize viral peptides generated intracellularly (83), it is unlikely that the virus could hide for long. Another interesting possibility is that the lipid composition of viral envelopes provides an advantage. The lipid composition of the ERGIC is distinct from that of the plasma membrane (24), and this difference is reflected in the lipid composition of coronavirus envelopes (24, 187). This distinct lipid composition could promote budding, virus-cell fusion, and/or stability of virions in different environments in the host.

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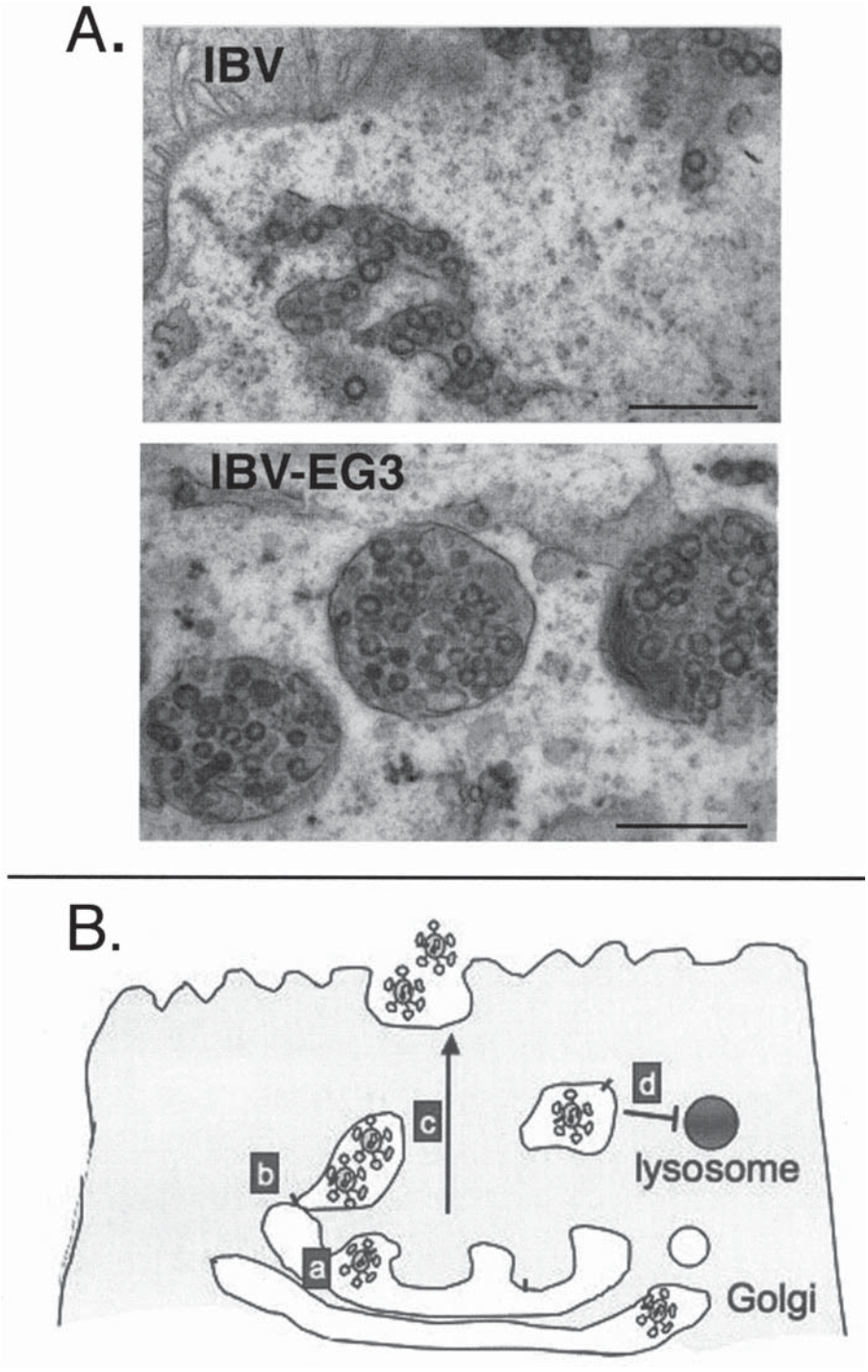

Figure 4. Potential roles for the E protein transmembrane domain in release of infectious virus. (A) Electron micrographs of Vero cells infected for 14 h with IBV or IBV containing an E protein with a heterologous transmembrane domain (IBV-EG3). Typical pleomorphic transport intermediates are present in cells infected with wild-type IBV, but large spherical vacuoles containing virions and degraded material are prominent in cells infected with IBV-EG3. Bars, 500 nm. (B) Results from mutations in the E protein transmembrane domain suggest that this domain could promote maturation of virions in late Golgi or post-Golgi compartments (a), promote formation of virus containing transport intermediates (b), promote fusion of transport intermediates with the plasma membrane (c), or prevent fusion of transport intermediates with lysosomes (d). The three last roles could be as a nonstructural protein. Ion channel activity or other interactions of the E protein transmembrane domain could be involved.

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Coronaviruses or VLPs with altered lipid compositions will be required to test these ideas.

It remains to be determined what components actually initiate coronavirus budding. It was initially assumed that M-nucleocapsid interactions drive the budding process, with the long cytoplasmic tail of M functioning like a "receptor" for the nucleocapsid. M-nucleocapsid interactions do occur, and it is likely these interactions drive budding of nucleocapsids into the virion envelope. Do the M protein tails form a matrix-like structure along the membrane that ultimately lines the inner virion envelope (like matrix proteins of negative-strand viruses) to facilitate budding? The structure of coronaviruses should be more extensively investigated to determine if association of M protein with the nucleocapsid to form a core is a common characteristic of these viruses. This will require careful state-of-the-art microscopy studies of morphogenesis and of virions. Does the M protein play a role in encapsidation? Studies that address whether coronavirus RNA encapsidation is mediated by interactions between N and M proteins or directly between the M protein and RNA genomic packaging signals should answer this question.

At a fundamental level it is clear that the envelope can assemble in the absence of nucleocapsids, since coexpression of M and E proteins is sufficient for formation of VLPs, which resemble "spikeless" virions lacking a genome. The function(s) of the coronavirus E protein in assembly is still a mystery. Does it induce curvature of membranes containing a scaffold of the M protein, or promote the scission step at the conclusion of budding? Is the proposed hairpin topology conformation of E protein the form that promotes virus assembly? One important issue is how much E protein is actually incorporated into virions. Is there a fixed ratio of M to E protein in virions? This would be expected if E protein is required to perturb an M scaffold at precise places to induce curvature. If E protein does promote assembly, how do MHV and SARS-CoV replicate in its absence?

It will also be important to examine the role of the putative ion channel activity in assembly and release of infectious virions. Further experiments to define the sequence requirements of the transmembrane domain are essential. Is the ion channel required in assembled virions, or as a nonstructural protein in Golgi or post-Golgi membranes? If this activity is required in virions, does it promote "maturation" observed for some coronaviruses (which could be required for subsequent stability of virions)? One important piece of the puzzle that is missing is the localization of E protein at the electron microscopic level in infected cells. Knowing if E protein moves past the Golgi complex late in infection should help define the compartment in which a potential nonstructural role may be required. If E protein promotes efficient release of infectious virus, why is TGEV absolutely dependent on its expression, whereas MHV and SARS-CoV are not?

Finally, nothing is known about the role of host proteins in coronavirus assembly. Human immunodeficiency virus and other enveloped viruses co-opt cellular machinery for budding (140). It is likely that cellular factors are recruited to assist with coronavirus envelope biogenesis and budding, but these have not been identified and the processes in which they may be involved are unknown at this time. This is an area that requires significant investigation.

The emergence of SARS-CoV sparked tremendous interest in and recognition of coronaviruses as intriguing for their molecular and cellular biology and as significant pathogens. As a result, the number of investigators involved in research on coronaviruses has significantly increased, which has brought new ideas and experience to the field. The availability of new tools such as infectious clones provides the opportunity to use reverse genetics to address many important questions. Answers to many of the remaining issues regarding coronavirus assembly should be forthcoming, which will no doubt raise new interesting questions to ponder and investigate.

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