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Visions & Reflections (Minireview)

Coronavirus envelope protein: A small membrane protein with multiple functions

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Abstract. Coronavirus envelope protein is a small membrane protein and minor component of the virus particles. It plays important roles in virion assembly and morphogenesis, alteration of the membrane permeability of host cells and virus-host cell interaction. Here we review recent progress in characterization of the biochemical properties, membrane topology and functions of the protein.

Keywords. Coronavirus, envelope protein, biochemical properties, membrane topology, virion assembly, viroporin, apoptosis.

Introduction

Coronaviruses are enveloped viruses with a single-strand, positive-sense RNA genome of 27–32 kb in length. In coronavirus-infected cells, a 3'-co-terminal nested set of six to nine mRNA species, including the genome-length mRNA (mRNA1) and five to eight subgenomic mRNA species (mRNA2–9), is produced. The four major structural proteins, spike (S), envelope (E), membrane (M) and nucleocapsid (N), are encoded by different subgenomic mRNAs. Similar to most of other RNA viruses, the genomic RNA replication, mRNA transcription and protein synthesis of coronavirus occur in the cytoplasm of the infected cells. The newly synthesized structural proteins and the RNA genome are assembled into virions that bud at the ER-Golgi intermediate compartment [1, 2]. Following translocation to the Golgi apparatus for further modification and maturation, the mature particles are released from the infected cells through the secretary pathway.

Coronavirus E protein is a small envelope protein present in virions at low levels, and ranges in size from 76 to 109 amino acids [3–5]. The E proteins from infectious bronchitis virus (IBV) and mouse hepatitis virus (MHV) are translated from the third and second ORFs of mRNA 3 and 5 of the respective viruses by a cap-independent, internal ribosomal entry mechanism [6–12]. In some coronaviruses, *e.g.*, in severe acute respiratory syndrome coronavirus (SARS-CoV), the E protein is derived from a monocistronic mRNA [13]. There is a low degree of sequence identity among E proteins from three coronavirus groups, and in some cases, among members of the same group (Fig. 1). Nevertheless a number of general features can be highlighted in all coronavirus E proteins. These include a short hydrophilic N-terminal region, followed by a long hydrophobic stretch of 21–29 amino acid residues, 2–4 cysteine residues

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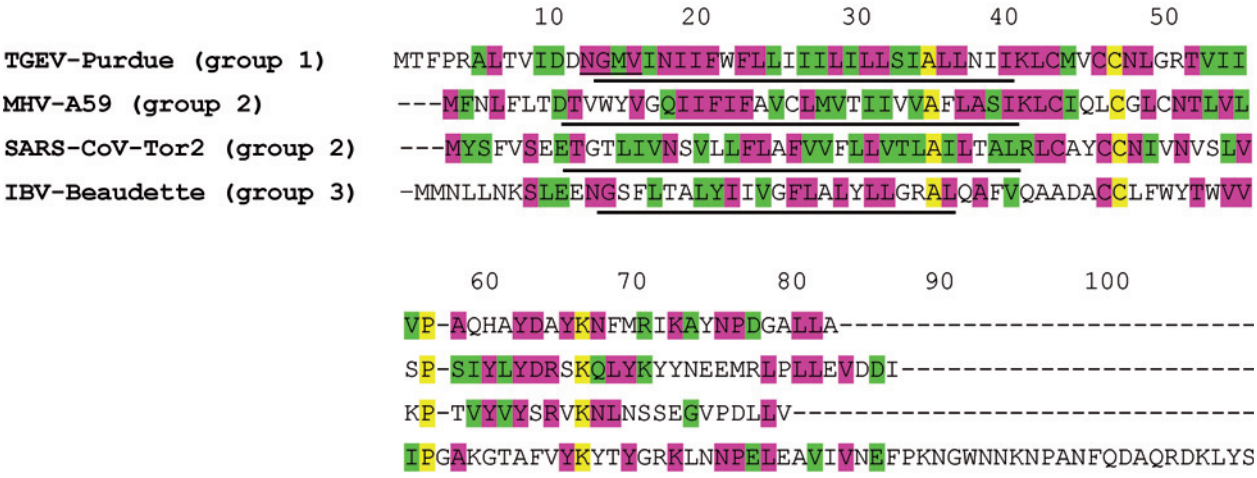

Figure 1. Alignment of the amino acid sequences of the E protein encoded by transmissible gastroenteritis virus (TGEV), mouse hepatitis virus (MHV)-A59, severe acute respiratory syndrome coronavirus (SARS-CoV) and infectious bronchitis virus (IBV). The putative transmembrane domains are underlined, the identical residues are highlighted in *yellow*, the conserved residues are highlighted in *pink* and the similar residues are highlighted in *green.*

immediately downstream of the hydrophobic stretch, and a relatively long hydrophilic C-terminal tail (Fig. 1).

Subcellular localization, biochemical properties and membrane topology of coronavirus E protein

In cells infected with some coronaviruses, such as IBV, MHV and bovine coronavirus (BCoV), the E protein exhibits a granular or punctuate pattern on immunofluorescence staining [5, 14, 15] and a portion of the E protein was transported to the cell surface [3, 5, 15]. The IBV E protein was shown to be localized to the Golgi complex when expressed on its own, coexpressed with M protein and in IBV-infected cells [16]. The C-terminal tail of this protein directs the Golgi targeting [17]. The SARS-CoV and IBV E proteins may be also transiently localized to the ER at early stages of the infection cycles [18–20]. The MHV E protein was found to accumulate in the ER-Golgi intermediate compartment as judged by their co-labeling with antibodies against E protein and Rab-1 [21].

Biochemical characterization of coronavirus E protein showed that the protein may undergo post-translational modifications. For example, the IBV and SARS-CoV E proteins are modified by palmitoylation on one or more cysteine residues [17, 18]. However, attempts to label the MHV and transmissible gastroenteritis virus (TGEV) E proteins with ^{3}H-labeled palmitic acid were unsuccessful [3, 21]. It is still unknown whether this modification is a general characteristic of the coronavirus E protein. In addition to palmitoylation, a minor proportion of the SARS-CoV E protein was recently found to be modified by N-linked glycosylation on Asn66 [22]. This modification is unique to SARS-CoV E protein, and it is still unknown whether the modification can also be detected in virus-infected cells and in virions. Coronavirus E protein can form homo-oligomers [23]. Molecular dynamic simulations predict that SARS-CoV E forms pentamers [23], but the oligomeric status of other coronaviruses is still unknown.

As mentioned earlier, different coronavirus E proteins share significant similarities in their biochemical properties as well as biological functions. However, they seem to adopt distinct membrane topology. Early studies showed that cell surface staining could be detected in cells infected with TGEV using C-terminal-specific antibodies against the TGEV E protein, suggesting that the C-terminal region of the protein is exposed to the external surface of the cells [3]. Immunofluorescence staining and biochemical analysis of cells expressing MHV E protein revealed that the C-terminal region is exposed to the cytoplasm and the N-terminal region is probably buried in the membrane near the cytoplasmic side ($N_{endo}C_{endo}$) [21, 24]. This model is supported by the observation that treatment of MHV particles with proteinase K did not alter the molecular mass of the E protein [21]. Consistent data on the topologies of MHV E protein in virions, in cells overexpressing the protein and in the *in vitro* translation systems in the presence of microsomal membranes have been reported [21, 24]. In the case of IBV E protein, immunofluorescence assay showed that C-terminal-specific antibodies could detect the protein in cells treated with either digitonin or Triton X-100, but the N-terminal-specific antibodies can detect the protein only in cells treated

with Triton X-100, demonstrating that the IBV E protein adopts an $N_{exo}C_{endo}$ topology [16]. This $N_{exo}C_{endo}$ topology was confirmed by the proteinase K protection assay [22].

Recently, the biophysical properties and membrane topology of the SARS-CoV E protein were studied by several groups [22, 23, 25–27]. Based on *in vitro* biophysical studies, Arbely and coworkers [25, 26] proposed an unusual short, palindromic transmembrane helical hairpin for the putative transmembrane domain of the protein. However, a regular α-helical structure with the N-terminal and C-terminal regions in opposite sides of the lipid bilayer has also been proposed based on similar *in vitro* biophysical studies and *in silico* data [23, 27]. The reason for this discrepancy is not clear. One possibility is that SARS-CoV E protein may adopt more than one topology. In fact, evidence for the presence of mixed membrane topologies for the SARS-CoV E protein has recently been reported [22]. Immunofluorescence analysis and proteinase K protection assay demonstrated that the majority of the SARS-CoV E protein adopts an $N_{endo}C_{endo}$ topology. Intriguingly, an N-linked glycosylation on Asn66 was found within the C-terminal region of the protein, confirming that a minor proportion of the SARS-CoV E protein is exposed to the luminal side. However, the membrane topologies of SARS-CoV E protein in virions and in virus-infected cells are still unknown. Considering the multiple roles of the coronavirus E protein in viral replication cycles, it would be of interest to relate different topology to each distinct function.

Multiple functions of coronavirus E protein

Pivotal roles in virion assembly and morphogenesis

Coronavirus E protein is crucial for virion assembly. Co-expression of M and E proteins, but not expression of M protein alone, led to the formation of virus-like particles (VLP), which are morphologically indistinguishable from coronavirions [16, 19, 28–31]. Additionally, expression of E protein on its own results in the release of E-containing membrane vesicles [32]. It is generally believed that E protein is required for the formation of coronavirus VLP. However, SARS-CoV M and N proteins were reported to be necessary and sufficient for the formation of VLP [33], although this is not consistent with a report showing that SARS-CoV M and E are sufficient for VLP formation [30]. It either indicates that SARS-CoV might adopt a unique mechanism for virion assembly, or simply suggests that formation of VLP may vary with the expression systems used. The precise function of E protein in virion assembly is not fully elucidated, but its low abundance in virions and in VLP implied that it might serve to induce membrane curvature or act to pinch off the neck of the viral particles at the final stage of the budding process. Analysis of BHK cells expressing the MHV E protein by electron microscopy showed that the protein could induce the formation of tubular structures in the ER-Golgi intermediate compartment network, suggesting that this protein has a tendency to induce membrane curvature [21]. *In vitro* incorporation of the transmembrane domain of SARS-CoV E protein into lipid vesicles can also deform the vesicle and induce changes in the thickness of the lipid bilayer and acyl-chain ordering [25, 26]. More recently, the transmembrane domain of the IBV E protein was shown to be required for efficient release of virus particles [34].

Coronavirus E protein is also involved in the morphogenesis of viral particles. Mutations introduced into the C-terminal hydrophilic tail of MHV E protein by targeted RNA recombination showed that one of the mutants was remarkably thermolabile and appeared to be aberrant and heterogeneous in virion morphology, exhibiting pinched and elongated shapes instead of the normal rounded shapes [35]. This phenotype suggests that E protein is essential for creating the membrane curvature needed to acquire the rounded and stable virions. However, a more authoritative and in-depth study of the virion structure and morphology is needed to exclude the possibility that the observed abnormal morphology of the mutant virus may be caused by artificial factors during sample preparation, as the mutant virus might be more fragile than wild-type virus. A mutant MHV with deletion of the E gene was recovered later, although the mutant virus produces tiny plaques and shows low growth rate and titer [36]. Alanine scanning insertion mutagenesis of the hydrophobic domain of the MHV E protein suggested that positioning of polar hydrophilic residues within the predicted transmembrane domain is important for virus production [37]. Substitution of the MHV E gene with heterologous E genes from viruses spanning all three groups of coronavirus family showed that the E proteins of group 2 and 3 coronaviruses could almost fully replace the MHV E protein [38]. However, E protein of the group 1 coronavirus, TGEV, could functionally replace the MHV E protein only after acquisition of particular mutations [38]. As these E proteins share a low degree of sequence identity, it indicates that sequence-specific contacts with other viral components may not be essential. More recently, a recombinant SARS-CoV that lacks the E gene was rescued in Vero E6 cells, and the recovered deletion mutant was attenuated in *vitro* and in the hamster model [39]. Interestingly,

electron microscopy analysis showed that wild-type and the deletion mutant viruses are morphologically identical [39]. At variance for group 1 TGEV, deletion of E protein is lethal, as shown in two independent reverse genetic experiments [40, 41]. In general, coronavirus E protein is not essential for viral replication, but seems to be a non-obligate budding enhancer.

An intriguing question on coronavirus assembly is whether direct interaction between E and M proteins is required for virion assembly. Physical interaction of the two proteins was demonstrated by co-immunoprecipitation in virus-infected or transfected cells [19, 32, 42]. However, studies of IBV E and M mutants showed that interaction between the two proteins is not sufficient for the VLP formation [42]. Meanwhile, chimeric M protein was able to form VLP with TGEV E, BCoV E, and BCV-TGEV chimeric E proteins [28, 42]. As mentioned above, the E proteins of group 2 and 3 coronaviruses were readily inter-changeable for that of MHV. These results suggest that sequence-specific contacts of E protein with M protein may not be essential for virion assembly.

Induction of apoptosis

Two coronavirus E proteins, MHV and SARS-CoV E proteins are apoptosis inducers [43, 44]. The apoptotic pathway induced by MHV E protein can be blocked by overexpression of Bcl-2, suggesting that the initiation of the apoptotic pathway by this protein is upstream of Bcl-2 [43]. SARS-CoV E protein was also able to induce apoptosis in the transfected Jurkat T cells, which can be inhibited by overexpression of Bcl-xL [44]. A BH3-like domain located in the C-terminal region of the SARS-CoV E protein could mediate binding of the protein to Bcl-xL [44]. However, induction of apoptosis does not appear to be a general feature of the coronavirus E protein and is cell-type specific. For example, MHV infection induced apoptosis in 17Cl-1 cells but not in DBT cells [43]. Constitutive expression of the TGEV E protein in BHK cells using a Sindbis replicon system did not induce apoptosis [41]. However, apoptosis induced by coronavirus E protein was observed in cells overexpressing the protein. This may not faithfully reflect the real situation in virus-infected cells.

Ion channel activity

Several small viral membrane proteins, such as the influenza virus M2 protein and the HIV Vpu protein, could modify host cell membrane and form ion channels. Recently, the SARS-CoV E protein was shown to form membrane channels with selectivity for monovalent cations [45]. Moreover, a peptide corresponding to the N-terminal 40 amino acids of the SARS-CoV E protein had the same properties as the full-length E protein. Similar to other viroporins [46], expression of SARS-CoV and MHV E protein enhanced the membrane permeability of bacterial and mammalian cells [47, 48]. This channel-forming activity of coronavirus E protein was more recently extended to the E proteins of human coronavirus 229E (HCoV-229E), MHV, and IBV [49], and may therefore be a general feature of the E protein from all three groups of coronavirus. Interestingly, channels formed by E proteins of group 2 (MHV and SARS-CoV) and group 3 (IBV) coronaviruses show greater preference for sodium ions over potassium ions [45, 49]. By contrast, the ion channels formed by the E protein of group 1 coronavirus HCoV-229E exhibit greater preference for potassium ions over sodium ions [49].

Hexamethylene amiloride (HMA), an amiloride analogue, blocks the ion channel activity of HIV Vpu [50, 51], hepatitis C virus (HCV) P7 [52] and dengue virus M protein [53]. This molecule could also inhibit the ion channel activity of the HCoV-229E and MHV E proteins, but not the IBV E protein [49], suggesting a more divergent structure of group 3 coronavirus E protein. Furthermore, HMA is able to inhibit the replication of HCoV-229E and MHV, but not the replication of a recombinant MHV with deletion of the entire E gene [49]. These results indicate that the ion channel activity of coronavirus E protein is important for virus replication, especially in the case of some coronaviruses, such as MHV.

However, the exact role of E protein in coronavirus life cycle is yet to be revealed. It is believed that small ion channels formed by virus-encoded proteins can help uncoating or release of mature virus particles. For example, the influenza virus M2 protein can form a proton channel to lower the interior pH of the virion. The lower internal virion pH is also thought to weaken protein-protein interactions between the viral matrix protein, the ribonucleoprotein core and the lipid bilayer, thereby freeing the viral genome from interactions with viral proteins and enabling the viral RNA segments to migrate to the host cell nucleus. One possibility of how E protein could enhance the release of the mature viral particles is that dissipation of the ionic gradient in the ER-Golgi intermediate compartment as well as the Golgi compartment may promote virion exit through the transport pathway. Further exploration of the functional significance of E protein in coronavirus life cycles may reveal more detailed information of coronavirus replication mechanisms and pathogenesis.

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