THE TRANSMEMBRANE DOMAIN OF THE INFECTIOUS BRONCHITIS VIRUS E PROTEIN IS REQUIRED FOR EFFICIENT VIRUS RELEASE

Carolyn E. Machamer and Soonjeon Youn*

1. INTRODUCTION

The envelope protein (E) of coronaviruses plays an important role in virus assembly, even though it is only incorporated at low levels into virions. Virus-like particles (VLPs) are produced when the membrane (M) protein and E protein are co-expressed, but not when M is expressed alone. Thus, the E protein may help to induce membrane curvature at precise places within a scaffold made up of the M protein. Using coronavirus infectious clones, it was shown that the transmissible gastroenteritis virus E protein is essential for virus production, and murine hepatitis virus lacking E protein is viable but extremely debilitated.

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Some enveloped viruses including influenza and human immunodeficiency virus encode small membrane proteins that form ion channels in infected cells. The E protein of the severe acute respiratory syndrome (SARS) coronavirus has recently been shown to form a cation-specific ion channel in synthetic membranes. This observation suggested that we reevaluate the IBV E mutant with a substituted transmembrane domain. Because the pore for ion movement forms from the transmembrane segments of ion channels, replacing the sequence would be expected to block channel function. After replacing the wild-type E protein sequence for that of the transmembrane-substituted E protein in an infectious clone for IBV, we recovered and characterized the

*Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.
recombinant virus. Our results suggest that the amino acid sequence of the transmembrane domain is important but not essential for production of infectious virus.

2. METHODS

The cDNA encoding EG3 was used as a template to introduce the mutant E sequence into a molecular clone of the Beaudette strain of IBV. In vitro ligation, transcription of RNA, electroporation, and recovery of virus in Vero cells were as previously described. Virus was plaque-purified and after amplification, RT-PCR sequencing confirmed the mutation. Two independent clones were used for the characterization of IBV-EG3. Purified virus was examined by negative stain electron microscopy on parlodian-coated grids stained with phosphotungstic acid. Thin section EM was performed after Epon embedding on osmium tetroxide stained samples as previously described. IBV antibodies and immunoblotting have also been described.

3. RESULTS

Because replacing the transmembrane domain of the IBV E protein had no effect on targeting or assembly of VLPs in transfected cells, we had an unprecedented opportunity to ask if the E transmembrane domain had another function in the context of a virus infection. Using a recently developed infectious clone for IBV, we replaced the sequence of E with that for EG3, a mutant IBV E that contains the transmembrane domain sequence of a heterologous membrane protein (VSV G). Infectious virus was recovered, indicating that the amino acid sequence of the E transmembrane domain was not essential for the virus replication cycle. We first characterized the mutant IBV-EG3 for its growth properties. In a single step growth curve, IBV-EG3 reached a peak titer later than the wild-type virus, and produced 10-fold less total infectious virus (Fig. 1A). In addition, there was a significant defect in release of infectious virus when the supernatants and cells were titered separately (Fig. 1B). At 14 h post-infection, cells infected with IBV-EG3 released about 200-fold less infectious virus into the supernatant compared to cells infected with IBV.

We showed that the EG3 protein interacted with IBV M as well as the wild-type E protein in infected cells, and was targeted and modified with palmitate normally (data not shown). We also found that entry and early stages of virus replication appeared normal. Due to the reduced release of infectious virus, we compared the virus particles produced in cells infected with IBV-EG3 to those from cells infected with wild-type IBV.
Figure 1. (A) Single-step growth curves for IBV and IBV-EG3. Vero cells were infected with wild-type IBV or two different clones of IBV-EG3 (8 and 10) at a multiplicity of infection of 4. Total virus was harvested at the times indicated by freezing the supernatant and cells together. Titers were determined by plaque assay on Vero cells. (B) The supernatants and cells were harvested separately from cells infected with IBV or IBV-EG3 at 14 h and 24 h postinfection. The percent release was calculated from the total infectious virus in cells plus supernatant at each time point.

Although infectious virus was significantly lower in supernatants of cells infected with IBV-EG3 relative to IBV, particle production was only decreased about 50%. By negative stain electron microscopy, most of the IBV-EG3 particles appeared defective, with absent or reduced spikes (Fig. 2). By contrast, wild-type IBV had the typical coronavirus appearance. When we examined the polypeptide content of purified particles, there was a significant reduction of S protein by immunoblotting (Fig. 3). Most of the S protein appeared to be cleaved from the particles, with only the internal cytoplasmic tail and transmembrane domain remaining.

Finally, we examined infected cells by thin section EM to determine if particles accumulated in cells infected with the mutant IBV-EG3. Budding profiles and virions inside Golgi membranes were observed for both viruses (Fig. 4). However, cells infected with IBV-EG3 contained more vacuoles filled with virions compared to those infected with wild-type IBV (Fig. 4). In some cases, the vacuoles in IBV-EG3 infected cells appeared to be autophagosomes, because degenerating organelles could also be found inside them.

4. DISCUSSION

The results presented here support the idea that the transmembrane domain of the coronavirus E protein plays an important role in a late stage of the virus replication cycle. Particles purified from the supernatants of IBV-EG3-infected cells lacked a full complement of spikes and contained what appeared to be a small, C-terminal proteolytic fragment of the spike protein. Thus, although the transmembrane domain of the IBV E protein is not required for formation of virus particles, it does appear to be required for their efficient release in an infectious form.
Figure 2. IBV-EG3 particles are defective. Negative staining was performed on particles purified on sucrose gradients from cells infected with IBV or IBV-EG3. Most of the IBV-EG3 particles lacked a full complement of spikes, whereas IBV had the normal coronavirus appearance. Bars, 100 nm.

Figure 3. Biochemical analysis of purified particles. Purified particles were electrophoresed in 10% (S and N) or 15% (M and E) polyacrylamide-SDS gels, transferred to Immobilon membrane, and immunoblotted for IBV S, N, M or E proteins. IBV-EG3 lacked full-length S protein, and instead contained a small fragment that reacted with an antibody recognizing the C-terminus of IBV S (arrowhead). In addition, about 50% of the M protein lacked the N-terminal domain (asterisk).
If the coronavirus E protein forms a cation-specific ion channel, we predict that the EG3 protein would lack such an activity. Because a recombinant IBV containing the chimeric E protein could be isolated, such an activity is not essential for the virus. However, the putative ion channel activity could be important for protecting virus-containing secretory vesicles from fusion with lysosomes. The exact mechanism of coronavirus release after budding into the endoplasmic reticulum–Golgi intermediate compartment is not known. Large vacuoles containing budded virions are observed in infected cells, presumably en route to the plasma membrane where fusion of the vacuole results in release of virions.\textsuperscript{14} Perhaps the excess E protein produced in infected cells functions to modify membrane traffic pathways to enhance fusion of virus-containing vacuoles with the plasma membrane rather than with lysosomes. This could either be by modification of microenvironments via ion channel activity or by interaction with host cell membrane trafficking machinery directly. Fusion of vacuoles containing partially degraded virions with the plasma membrane would release these particles into the supernatant, explaining the biochemical and morphologic appearance of IBV-EG3. Regardless of whether the E protein forms an ion channel or interacts directly with membrane traffic machinery, the transmembrane domain of the E protein appears to play an important role in this second, nonstructural role in the virus replication cycle. Future experiments will be directed towards understanding the role of the E transmembrane domain in coronavirus release.
5. ACKNOWLEDGMENTS

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6. REFERENCES

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